PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/87, 15/10 — A1

(11) International Publication Number: **WO 00/26396**

(43) International Publication Date: 11 May 2000 (11.05.00)

(21) International Application Number: PCT/AU99/00835

(22) International Filing Date: 29 September 1999 (29.09.99)

(30) Priority Data:
PP 6849 30 October 1998 (30.10.98) AU

(71) Applicant *(for all designated States except US)*: THE MURDOCH INSTITUTE [AU/AU]; 10th floor, Royal Children's Hospital, Flemington Road, Parkville, Victoria 3052 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: IOANNOU, Panos [GB/AU]; 442 Brunswick Road, West Brunswick, Victoria 3055 (AU). NARAYANAN, Kumaran [MY/MY]; Faculty of Research Science and Technology, Univisiti Malaysia Sarawak, Kota Famarahan, 94300 Sarawak (MY).

(74) Agents: DE BRUYN, Paula, J. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, Victoria 3000 (AU).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: A METHOD OF RECOMBINATION AND AGENTS USEFUL FOR SAME

(57) Abstract

The present invention relates generally to a method for the recombination of at least two nucleic acid sequences and agents useful for same. More particularly, the present invention contemplates a method of recombining, in a host cell which expresses the *recBCD nuclease* or a functional derivative thereof, a circular nucleic acid sequence with a linear nucleic acid sequence. The method of the present invention is useful, *inter alia*, for the modification of bacterial artificial chromosomes by homologous recombination with a linear DNA sequence.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

A METHOD OF RECOMBINATION AND AGENTS USEFUL FOR SAME

FIELD OF THE INVENTION

The present invention relates generally to a method for the recombination of at least two nucleic acid sequences and agents useful for same. More particularly, the present invention contemplates a method of recombining, in a host cell which expresses the *recBCD nuclease* or a functional derivative thereof, a circular nucleic acid sequence with a linear nucleic acid sequence. The method of the present invention is useful, *inter alia*, for the modification of bacterial artificial chromosomes by homologous recombination with a linear DNA sequence.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

There is a need for a greater understanding of the structure and organisation of the genomes of higher eukaryotes. Genomic sequences are oftern much longer than coding sequences. Although the functions of most non-coding sequences are not known, it is clear that may play a crucial role in regulation of gene expression, stability and evolution. In contrast to cDNA constructs driven by unrelated or viral promoters, gene transfer of large genomic fragments shows correct temporal- and tissue-specific gene expression. [1-7]

Yeast artifical chromosomes (YACs) have provided genomic clones of many disease genes. [8-10] Transgenic models with YACs for a number of human genetic loci have demonstrated the usefulness of such large genomic fragments to produce accurate models for various diseases. [11-14] The high efficiency of homologous recombination in yeast has been of crucial significance in the genetic manipulation of YACs for functional analysis, [3,8,15] the circularisation of YACs into PACs or BACs [16,17] and the generation of larger YACs from smaller overlapping YACs. [18]

There are, however, several limitations with the YAC system which have seriously impeded its use. YACs have a high degree of clonal instability and chimerism. [19,20] Furthermore, although a number of methods have been developed to generate YAC transgenics, purification of intact YAC DNA for transgenesis is difficult. [21] An important disadvantage of the yeast homologous recombination system for introducing modifications in YACs or for TAR cloning, [22] is the difficulty in regulating the extent of homologous recombination and the need to screen large numbers of "recombinant" clones in order to identify the correct products.

The large-insert BAC/PAC cloning systems [23,24] have overcome many of the difficulties of the YAC system. BACs/PACs are used increasingly for long-range physical mapping, [25,26] positional cloning of disease genes, [27] whole genome sequencing projects [28-30] and functional studies. [31,32] High quality BAC/PAC genomic libraries are much easier to construct than YAC libraries because of greater cloning efficiency in bacteria. BACs/PACs are maintained at 1-2 copies per cell in a well-defined recombination-deficient *E. coli* strain, DH10B, where they exhibit high clonal stability over many generations. Although they carry inserts up to about 300kb in size, they can be purified in large quantities for functional studies through conventional bacterial plasmid isolation methods. The genomic inserts in these clones are large enough to preserve the integrity of most human genetic loci and are thus ideal for functional studies.

Despite these advantages and the increasing popularity of BACs/PACs as important tools for genome studies in many systems, there are no convenient techniques for performing genetic manipulations on these clones. The large size of the genomic inserts in these clones precludes the finding of convenient restriction sites on a routine basis.

Homologous recombination in an F-plasmid-based vector in *E. coli* was first used in 1989 to join overlapping Drosophila cosmid fragments to form a 125kb fragment. [35] Co-integration between a shuttle plasmid with a temperature-sensitive origin of replication in a rec^+ strain, while a second site-specific recombinational event in a rec^- host was needed to resolve the co-integrates. Sternberg[34] described a method for the transpositional

insertion of a eukaryotic selectable marker into large P1 clones, which should also be applicable to PACs and BACs, although it may be difficult to control precisely the site of transposition. The RecA-assisted restriction endonuclease (RARE) cleavage approach has also been used successfully to introduce modifications in a number of P1 and BAC clones, [35] although it is technically a very restrictive procedure. Since both the PAC and BAC cloning systems include a *lox*P site on the backbone of each vector, a number of workers have targeted the insertion of eukaryotic selectable markers and reporter genes into this site with *cre* recombinase.[36,37]

Yang *et al* [38] reported targeted modification of a 131kb BAC using the wild type *rec*A gene in a shuttle plasmid with a temperature-sensitive origin of replication, as a means of conferring transient recombination proficiency to DH10B cells. There was evidence, however, for significant rearrangements of clones using this procedure, necessitating characterisation of recombinant clones by Southern blot to identify the correct products. An alternative approach using the CBTS strain of *E. coli*, which carries a temperature-sensitive amber mutation in the *rec*A gene, has also been used to introduce modifications in a 230kb BAC containing the intact mouse CMV genome.[39] However, direct transfer of BACs from DH10B to the conditional *rec*A strain is difficult. Recently the green fluorescent protein gene was targeted into a zebrafish BAC containing the *GATA-2* locus, using a targeting construct containing properly oriented chi sites. [40] This necessitated, however, the transfer of the BAC to a recombination proficient strain, which could lead to instability in clones with repetitive sequences.

A method has also been described for the replacement of *E. coli's* RecBCD function with bacteriophage λ's Red-Gam system. [41] This replacement allowed homologous recombination between the host chromosome and short linear DNA fragments to proceed at a rate that is at least 70-fold higher than that exhibited by a *recBC sbsBC* or *recD* strain. However, the usefulness of this system in the modification of BACs/PACs was not examined.

None of the above procedures is optimal for the genetic manipulation of PACs and BACs.

They cannot be applied directly in host cells, such as DH10B, which express *RecBCD* or require the construction of complex shuttling vectors. The extent of homologous recombination is also not well controlled is most of these approaches, thus running the risk of unwanted rearrangements. Accordingly, there is a need to develop methods of recombining two nucleic acid sequences, such as BAC's/PAC's with linear DNA sequences, directly in host cells in a controlled manner.

In work leading up to the present invention the inventors sought to develop a method for the controlled homologous recombination, *in vivo,* of two nucleic acid sequences such as a circular DNA sequence with a linear DNA sequence. In particular, the inventors have modified BAC's by expressing in a host cell, a modulatable recE/recT recombination system together with modulatable levels of a recBCD nuclease inhibitor thereby permitting the homologous recombination of a DNA sequence with the BAC. Expression of the recBCD nuclease inhibitor acts to inhibit the functional activity of endogenously produced recBCD nuclease. Further, the capacity to modulate and coordinate expression of both the recE/recT homologous recombination system and the recBCD nuclease inhibitor facilitates the control of homologous recombination events and minimises random and unwanted recombination events. The capacity to modulate and coordinate expression of the homologous recombination system and the recBCD nuclease inhibitor facilitates the, *in vivo*, induction of homologous recombination events both where at least one of the nucleic acid sequences which is the subject of recombination is located in an F-plasmid, such as a BAC, or where the host cell is sensitive to the over expression of recombination system molecules, or recBCD inhibitors such as gam.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography.

Accordingly, the present invention provides a method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

Another aspect of the present invention provides a method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD, recE, recT* and a nucleotide sequence encoding a recBCD inhibitor or functional derivatives thereof, the expression of which *recE, recT* and nucleotide sequence encoding a recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

Yet another aspect of the present invention provides a method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD*, *recE, recT* and *gam* or functional derivatives thereof, the expression of which *recE, recT* and *gam* is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

Still another aspect of the present invention provides a method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD*, *recE, recT* and *gam* or functional derivatives thereof, the expression of which *recE, recT* and *gam* is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

Still yet another aspect of the present invention provides a method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

A further aspect of the present invention provides a method for facilitating homologous recombination between a circular nucleotide sequence and a linear nucleotide sequence said method comprising inducing recombination between said circular nucleotide sequence and said linear nucleotide sequence in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the circular nucleotide sequence with the linear nucleotide sequence.

Another further aspect of the present invention provides a method for facilitating homologous recombination between a circular nucleotide sequence and a linear nucleotide sequence said method comprising inducing recombination between said circular nucleotide sequence and said linear nucleotide sequence in a DH10B cell or homolog or mutant

thereof which is capable of expression *recBCD*, *recE, recT* and *gam* or functional derivatives thereof, the expression of which *recE, recT* and *gam* is modulatable, by culturing the DH10B cell or homolog or mutant thereof under conditions sufficient to facilitate the homologous recombination of the circular nucleotide sequence with the linear nucleotide sequence.

Still another further aspect of the present invention provides a method for facilitating homologous recombination between at least two nucleotide sequences wherein at least one of said nucleotide sequences comprises an F-plasmid portion said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivative thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modultable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

Still yet another aspect of the present invention provides a method for facilitating homologous recombination between a BAC and a linear nucleotide sequence said method comprising inducing recombination between said BAC and said linear nucleotide sequence in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the BAC with the linear nucleotide sequence.

Another aspect of the present invention contemplates a method for facilitating the homologous recombination of at least two nucleotide sequences in a host cell said method comprising the steps of:

(i) transforming a host cell, wherein the transformed host cell comprises a first nucleotide sequence encoding an exonuclease, a recombination protein and a

recBCD inhibitor, or functional derivatives thereof, the expression of which exonuclease, recombination protein and gene encoding the recBCD inhibitor is modulatable, and said at least two nucleotide sequences; and

(ii) culturing said transformed host cell for a time and under conditions sufficient for expression of said first nucleotide sequence to occur wherein the expression products of said first nucleotide sequence facilitate the homologous recombination of said second nucleotide sequence with said third nucleotide sequence.

Still another aspect of the present invention contemplates a method of producing a microorganism useful for facilitating homologous recombination between at least two nucleotide sequences said method comprising genetically manipulating a host cell such that it is capable of expressing *recBCD*, modulatable levels of an exonuclease, a recombination protein and a gene encoding a recBCD inhibitor and said at least two other nucleotide sequences.

Still another further aspect of the present invention contemplates a cell capable of facilitating homologous recombination between at least two nucleotide sequences said cell comprising nucleotide sequences encoding an exonuclease, a recombination protein and a gene encoding a recBCD inhibitor, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, recBCD and said at least two nucleotide sequences.

Still yet another aspect the present invention contemplates nucleic acid molecules homologously recombined by the method of the present invention.

A further aspect of the present invention contemplates a pharmaceutical composition comprising modified nucleic acid molecules generated by the method of the present invention together with one or more pharmaceutically acceptable carriers and/or diluents.

Yet another further aspect of the present invention is directed to a kit for facilitating, in a host cell, the homologous recombination of at least two nucleotide sequences said kit comprising compartments adapted to contain any one or more of nucleotide sequences encoding an exonuclease, a recombination protein, a recBCD inhibitor or functional derivatives thereof, and reagents useful for facilitating homologous recombination. Further compartments may also be included, for example to receive biological samples such as any one or more of the nucleotide sequences which are to be recombined, the host cells or host cells already stably transformed with one or more of the nucleotide sequences which are to be recombined.

Still yet another further aspect of the present invention is directed to a kit for facilitating, in a host cell, the homologous recombination of at least two nucleotide sequences said kit comprising compartments adapted to contain any one or more of an exonuclease, a recombination protein, a recBCD inhibitor, or functional derivative thereof and reagents useful for facilitating homologous recombination. Further compartments may also be included, for example to receive biological samples such as any one or more of the nucleotide sequences which are to recombined, the host cell or host cells already stably transformed with one or more of the nucleotide sequences which are to recombined.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the pEBAC140 vector map, showing its main features. The vector is based on the backbone of the F-plasmid pBeloBAC11.[23] The hygromycin and thymidine kinase genes provide for selection in eukaryotic cells, while the *oriP* and *EBNA-1* genes from Epstein-Barr virus facilitate episomal maintenance. The vector contains a unique rare cutter multicloning site inserted in frame into the *lacZ* gene to allow for blue/white selection of recombinants and for recovery of genomic inserts by any of a number of different cutters which flank the *Bam*HI cloning sites. A modified pUC19-Xho is inserted into the *Xho*I site in later versions of the vector to render it multicopy and to facilitate cloning applications.

Figure 2 is a schematic representation of the pEBAC/148 which was generated by insertion of a 185kb genomic fragment from a PAC clone carrying the intact β-globin genomic region into the *Not*I sites of the pEBAC140 vector. The position of targeting the integration of the kanamycin resistance gene in this clone through homologous recombination and in the original pEBAC140 vector is indicated.

Figure 3 is a schematic representation of a map showing the main features of the pGETrec plasmid. A PCR product containing the *gam* gene of bacteriophage λ was inserted into the pBAD24-recET plasmid[45] to produce pGETrec. The *gam* gene is thus under the control of the arabinose promoter, together with the recE and recT genes.

Figure 4 is a schematic representation of the oligonucleotide primers used. PCRkan50 (1262bp) was generated by amplification of kanamycin gene from pCYPAC2 with primers kan50F and kan50R. Primers kan50F and kan50R also carry 50-mer targeting regions homologous to the sequences flanking the unique *Bst*1107I site on the vector target sequence. PCRkan140 was generated with primers KF and KR after homologous recombination of PCRkan50 into the pEBAC140 vector. The expected size of the PCR products with primers KF/KR is 288bp and 1450bp in the absence and presence of the kanamycin gene respectively. The expected size of the corresponding PCR products with

primers KA/KB is 393bp and 1555bp.

Figure 5 is a schematic representation of the "GET recombination" method for integration of PCR fragments through homologous recombination into BACs/PACs in DH10B cells. PCR primers are each designed to carry a 50-mer homology region to the targeted sites in a PAC or BAC, together with specific sequences at their 3'-ends for amplifying a DNA fragment with a selectable marker. Additional sequences or markers may also be included. The PCR product generated by these primers is electroporated into electrocompetent DH10B cells, carrying both the BAC or PAC clone of interest and the pGETrec plasmid, which are transiently induced with 0.2% L-arabinose during preparation. Recombination between the homology regions of the PCR fragment and the BAC or PAC clone allows integration of the PCR fragment. The modified BAC or PAC clone is purified away from pGETrec by miniprep DNA isolation and electroporation into DH10B cells or by streaking in media with an antibiotic.

Figure 6 is a photographic representation of PCR analysis of 20 independent Cm^rKm^r colonies carrying pEBAC/148::kan140. One microlitre of an overnight culture grown from each Cm^rKm^r colony was added into the PCR reaction with PCR primers KF and KR. The PCR products were resolved using a 1.5%(w/v) agarose gel. Lanes: 1 and 24, 1-kb DNA ladder standards; lanes 2-21, 20 independent Cm^rKm^r colonies; lane 22, parent pEBAC/148 clone; lane 23, negative control (no cells).

Figure 7 is a photographic representation of PFGE analysis of 4 randomly picked Cm^rKm^r colonies (A-D) after integration of PCRkan140 into pEBAC/148 by homologous recombination. DNA from each clone was re-electroporated into DH10B cells. Duplicate secondary colonies were used for miniprep extraction of DNA and restriction enzyme digestion with *Not*I or *Xho*I.

(a) *Not*I-digestion: M, low range PFG marker; Lanes 1-8, duplicate pEBAC/148::kan140 clones; P, parent pEBAC/148 clone. PFGE conditions: 6V/cm, switch time of 0.1-25.0s, 14°C, for 17 hours.

(b) *Xho*I-digestion: M1, low range PFG marker; Lanes 1-8, duplicate pEBAC/148::kan140 clones; P, parent pEBAC/148 clone; M2, midrange I PFG marker. PFGE conditions: 6V/cm, switch time of 0.1-8.0s, 14°C, for 18 hours.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated, in part, on the development of a method for the homologous recombination of at least two nucleotide sequences in a host cell by introducing into a host cell a nucleotide sequence which expresses modulatable levels of an exonuclease, a recombination protein and a recBCD nuclease inhibitor. Expression of a recBCD nuclease inhibitor is necessary to inhibit the linear DNA degradation activity of the recBCD nuclease which is endogenously expressed by host cells such as the *E. coli* strain DH10B. The method of the present invention is therefore based on the modulatable and coordinate induction of a homologous recombination system and the inhibition of recBCD nuclease activity. The advent of a modulatable and coordinate induction system has particularly facilitated the application of homologous recombination methodology in host cells which are sensitive to the over-expression of either one or more recombination system molecules or recBCD inhibitors. It is also particularly applicable in situations where the homologous recombination of a nucleic acid sequence comprising a F-plasmid portion is desired.

Accordingly, the present invention provides a method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

Reference to an "exonuclease" should be understood as a reference to a molecule which facilitates homologous recombination by exposing single stranded regions on a double stranded nucleic acid sequence which regions are necessary to effect recombination between this region and a complementary region of another nucleic acid sequence. The exonuclease is one which is suitable for use with the selected recombination protein.

Examples of exonucleases suitable for use in the present invention include, but are not limited, the Redλ recombination system exonuclease termed exo, and the recE/recT recombination system molecule exonuclease termed recE, or functional derivatives thereof. Preferably said exonuclease is recE or functional derivative thereof. Reference to a "recombination protein" should be understood as a reference to a peptide, polypeptide or protein which facilitates homologous recombination by mediating the recombination between the single stranded region of one nucleic acid sequence (such as the single stranded region generated by the exonuclease) and the complementary region of another nucleic acid sequence. Examples of the recombination protein include, but are not limited to, the Redλ recombination protein bet and the recE/recT recombination protein recT, or functional derivatives thereof. The nucleotide sequence encoding recE and recT are herein referred to as *recE* and *recT*, respectively.

Accordingly, the present invention more particularly provides a method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD, recE, recT* and a nucleotide sequence encoding a recBCD inhibitor or functional derivatives thereof, the expression of which *recE, recT* and nucleotide sequence encoding a recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

The term "expression" refers to the transcription and translation of a nucleotide sequence resulting in the synthesis of a peptide, polypeptide or protein.

Reference to "a gene encoding a recBCD inhibitor" should be understood as a reference to a nucleotide sequence which encodes an expression product which can either inhibit or sufficiently down-regulate the activity of the recBCD nuclease such that linear DNA sequences are not degraded by this nuclease and their homologous recombination is thereby able to occur. Examples of recBCD inhibitors suitable for use in the present invention include, but are not limited to, gam, P22Abc protein or SSB protein or

functional derivatives thereof . Preferably said recBCD inhibitor is gam. The nucleotide sequence encoding gam is herein referred to as *gam*.

According to this preferred embodiment, the present invention provides a method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD*, *recE, recT* and *gam* or functional derivatives thereof, the expression of which *recE, recT* and *gam* is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

Reference herein to "host cell" should be understood as a reference to any prokaryotic or eukaryotic cell which can be transformed or transfected with a nucleotide sequence. For example, contemplated herein are host cells suitable for cloning and/or expression of nucleotide sequences such as host cells which are used to create gDNA or cDNA libraries or those which are used for cloning a vector which comprises a DNA sequence insert of interest. In accordance with the present invention, the host cell is preferably one which expresses the recBCD nuclease (also termed "exonuclease V") which, *inter alia*, degrades linear, double stranded DNA. The host cell may be expressing *recBCD* either due to the expression of a naturally occurring recBCD gene or due to the transformation or transfection of the host cell with a nucleic acid molecule which encodes recBCD.

Without limiting the present invention to any one theory or mode of action, the inventors have determined that certain strains of host cell are sensitive to over-production, such as constitutive production, of a recBCD inhibitor and/or one or more molecules of a homologous recombination system. By "sensitive" is meant that the host cell is compromised in some way such as, for example, reduction in its viability or the undesirable modulation of one or more of its functional activities. For example, constitutive production of the recBCD inhibitor, gam, in DH10B host cells is found to be toxic in that the viability of DH10B cells is reduced. The development of an *in vivo* recombination system in which the expression of the recombination system molecules and

the recBCD inhibitor can be modulated and coordinated has overcome this toxicity problem. Accordingly, in a preferred embodiment the host cell is a host cell which is sensitive to the constitutive production of one or more of a recBCD inhibitor, an exonuclease or a recombination protein. Even more preferably said host cell is DH10B or mutant or homolog thereof.

The present invention extends to the use of mutants and homologs of host cells.

According to this most preferred embodiment, the present invention provides a method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

Even more preferably said exonuclease is recE, said recombination protein is recT and said recBCD inhibitor is gam.

Reference to "at least two nucleotide sequences" should be understood as a reference to any two or more nucleotide sequences which are to be homologously recombined by the method of the present invention. Those nucleotide sequences may be separate nucleic acid molecules such as, for example, two circular vectors (such as two plasmids), a plasmid and a linear DNA sequence or a chromosome and a linear DNA sequence. Alternatively, the nucleotide sequences may be two portions of a single nucleic acid molecule such as, for example, the linear and circular nucleotide sequence portions of the single nucleotide molecule generated during rolling circle replication. Preferably, the nucleotide sequences are a circular nucleotide sequence and a linear nucleotide sequence. In this context, a "circular" nucleotide sequence should be understood as a reference to the circular nucleotide sequence portion of any nucleotide molecule. For example, the circular

nucleotide sequence may be completely circular, such as a plasmid, or it may be partly circular, such as the circular portion of the nucleotide molecule generated during rolling circle replication. In this context, the "circular" nucleotide sequence corresponds to the circular portion of this molecule. Preferably, the circular nucleic acid sequence is a artificial chromosome of the type BAC or PAC. A "linear" nucleotide sequence should be understood as a reference to any nucleotide sequence which is in essentially linear form. The linear sequence may be a linear nucleotide molecule or it may be a linear portion of a nucleotide molecule which also comprises a non-linear portion such as a circular portion. Examples of linear nucleotide sequences include, but are not limited to, PCR products, excision products, synthesized DNA or the linear portion of a nucleotide molecule generated during rolling circle replication. Recombination of the linear nucleotide sequence with the circular nucleotide sequence may, for example, introduce into the circular nucleotide sequence a selectable marker or a specific mutation. It should also be understood that the homologous recombination of the present invention may occur between nucleotide sequences which are introduced into a cell or it may occur between nucleotide sequences which are naturally found in the cell and one or more introduced nucleotide sequences.

Accordingly, the present invention preferably provides a method for facilitating homologous recombination between a circular nucleotide sequence and a linear nucleotide sequence said method comprising inducing recombination between said circular nucleotide sequence and said linear nucleotide sequence in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the circular nucleotide sequence with the linear nucleotide sequence.

Preferably, said host cell is a DH10B cell. Even more preferably said exonuclease is recE, said recombination protein is recT and said recBCD inhibitor is gam or functional derivatives thereof.

According to this preferred embodiment, the present invention provides a method for facilitating homologous recombination between a circular nucleotide sequence and a linear nucleotide sequence said method comprising inducing recombination between said circular nucleotide sequence and said linear nucleotide sequence in a DH10B cell or homolog or mutant thereof which is capable of expression *recBCD*, *recE, recT* and *gam* or functional derivatives thereof, the expression of which *recE, recT* and *gam* is modulatable, by culturing the DH10B cell or homolog or mutant thereof under conditions sufficient to facilitate the homologous recombination of the circular nucleotide sequence with the linear nucleotide sequence.

The nucleic acid sequences of the present invention are preferably derivable from the human genome but genomes and nucleotide sequences from non-human animals and plants are also encompassed by the present invention. Non-human animals contemplated by the present invention include primates, livestock animals (eg. sheep, cows, pigs, goats, horses, donkeys), laboratory test animals (eg. mice, rats, guinea pigs, hamsters, rabbits), domestic companion animals (eg. dogs, cats), birds (eg. chickens, geese, ducks and other poultry birds, game birds, emus, ostriches) and captive wild or tamed animals (eg. foxes, kanagaroos, dingoes).

The inventors have determined that the homologous recombination system of the present invention is suitable for recombining at least two nucleotide sequences where at least one of said nucleotide sequences comprises an F-plasmid portion. Reference to a nucleotide sequence which "comprises" an F-plasmid portion should be understood as a reference to a nucleotide sequence which is fused, bound or otherwise associated with all or part of an F-plasmid. For example, the nucleotide sequence of interest may be located within a vector, the backbone of which comprises sequence corresponding to all or part of the F-plasmid. The F-plasmid based BAC for example comprises genomic sequences of interest which are located in a vector expressing the wild-type F-plasmid origin of replication and the par A, par B and par C encoding nucleotide sequences.

Accordingly, in another preferred embodiment the present invention provides a method for

facilitating homologous recombination between at least two nucleotide sequences wherein at least one of said nucleotide sequences comprises an F-plasmid portion said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivative thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modultable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

Preferably, said nucleotide sequence which comprises an F-plasmid portion is a BAC.

Even more preferably said host cell is DH10B or mutant or homolog thereof.

The BAC/PAC cloning system has facilitated the study of the mammalian genome via the gene transfer of large genomic fragments which show correct temporal and tissue-specific gene expression. Large-insert BAC/PAC cloning systems have therefore been used extensively for long-range physical mapping, positional cloning of disease genes, whole genome sequencing projects and functional studies. Accordingly, BAC/PAC *E. coli* libraries have been created for human genomic DNA as well as for the genomic DNA of other animal and plant species including baboon, canine, bovine, ovine, goat and rice. BACs/PACs are generally maintained at 1-2 copies per cell in the well defined recombination-deficient *E.coli* strain DH10B. Due to the interest in modifying BACs and PACs, by introducing linear DNA segments, a particularly preferred embodiment of the present invention is directed to the modification of a BAC or PAC via the homologous recombination of a linear DNA segment into the BAC or PAC.

According to this most preferred embodiment, the present invention provides a method for facilitating homologous recombination between a BAC and a linear nucleotide sequence said method comprising inducing recombination between said BAC and said linear nucleotide sequence in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or

derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the BAC with the linear nucleotide sequence.

Preferably, said host cell is a DH10B cell or mutant or homolog thereof. Even more preferably, said exonuclease is recE, said recombination protein is recT and said recBCD inhibitor is gam.

In yet another most preferred embodiment, the present invention provides a method for facilitating homologous recombination between a PAC and a linear nucleotide sequence said method comprising inducing recombination between said PAC and said linear nucleotide sequence in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the PAC with the linear nucleotide sequence.

Preferably, said host cell is a DH10B cell or mutant or homolog thereof. Even more preferably, said exonuclease is recE, said recombination protein is recT and said recBCD inhibitor is gam.

The method of the present invention may involve the transformation of the subject host cell with any one or more of *recBCD,* nucleotide sequences encoding an exonuclease and a recombination protein, a nucleotide sequence encoding a recBCD inhibitor and the at least two nucleotide sequences. How many of these molecules will require introduction into the host cell to facilitate the operation of the method of the present invention will depend on the particular application in issue. For example, with respect to the exemplified recE/recT recombination system it may be desirable to use a population of host cells from a commercially available BAC or PAC DH10B library. The BAC or PAC molecule may be modified by recombining it with a linear nucleotide sequence which is introduced into the commercially available DH10B clones from the library following tranformation with a

nucleic acid molecule encoding recE, recT and gam. DH10B cells endogenously express *recBCD*. Alternatively, the use of host cells which already express *recBCD*, *recE, recT* and a nucleotide sequence encoding a recBCD inhibitor will only require transformation with the nucleotide sequences which are to be homologously recombined and which are not already present in the host cell. These host cells may be expressing *recBCD, recE, recT* and a nucleotide sequence encoding a recBCD inhibitor either due to the cell having been engineered to express any one or more of these molecules or due to the cell endogenously expressing these molecules.

According to the method of the present invention, the expression of the exonuclease, the recombination protein and the recBCD inhibitor is modulatable. This permits the transient and coordinated expression of these proteins. By "modulatable" it is meant that the expression of the genes encoding these proteins can be up-regulated or down-regulated at the transcriptional or translation level. This modulation can be achieved by any one of a number of techniques including, but not limited to, regulating promoter expression or regulating methylation. For example, as exemplified herein, DH10B cells transformed with the BAC nuleic acid molecule, pEBAC140, are transfected with the inducible expression plasmid pGETrec which comprises the nucleic acid molecules encoding recE, recT and gam under the control of the inducible L-arabinose promoter. By culturing these cells in the presence of L-arabinose, expression of the pGETrec plasmid is induced. Subsequent electroporation of double stranded linear DNA, such as PCRkan140, into these cells results in the homologous recombination of PCRkan140 and pEBAC140 due to the operation of the recE/recT homologous recombination system and the simultaneous inhibition of recBCD nuclease activity by the *gam* expression product. Once homologous recombination has been completed, the subject DH10B cells can be removed from L-arabinose enriched culture medium thereby downregulating the expression of the pGETrec plasmid. This acts to shut down the activity of the recE/recT homologous recombination pathway.

Although the present invention is exemplified by the introduction, in a host cell, of a single plasmid which comprises the nucleic acid sequences *recE, recT* and *gam*, under the

control of an inducible promoter (i.e. the nucleic acid molecules encoding recE, recT and gam are operably linked to a common promoter), the method of the present invention should be understood to extend to the use of two or more vectors separately comprising *recE, recT* and *gam*. However, to achieve co-ordination with respect to the simultaneous expression of *recE, recT* and *gam* it would be necessary to ensure that the induction of expression of *recE, recT* and *gam* could be achieved simultaneously, for example, by utilising the same inducible promoter with each of *recE, recT* and *gam*. The recE/recT homologous recombination system would therefore become functionally active simultaneously with the gam inhibition of recBCD nuclease activity.

Reference to "facilitating" homologous recombination should be understood as a reference to inducing, enhancing or otherwise contributing to the functional operation of the homologous recombination system. For example, the *recE* and *recT* expression products induce recE/recT based recombination while the *gam* expression product inhibits the activity of the recBCD nuclease, which degrades linear DNA thereby preventing its recombination.

Without limiting the present invention to any one theory or mode of action, pGETrec can co-exist stably with BAC/PAC in *E. coli* DH10B cells. Utilising pGETrec, homologous recombination at high efficiency can be achieved between linear nucleic acid fragments and target circular nucleic acid molecules. Efficiency of homologous recombination is improved where the length of homology is increased from 50bp to 140bp. Further, it is thought that significant increases in efficiency of homologous recombination with BAC/PAC can be achieved by use of non-adjacent sequences in the targetted homology region.

The present invention should be understood to extend to the induction of *in vivo* homologous recombination by introducing into the host cell the proteins or functional derivatives thereof which facilitate homologous recombination rather than introducing into the host cell nucleic acid molecules which are induced to express these proteins. For example, the coordinated introduction of recE, recT and gam proteins, or functional

derivatives thereof, into the host cell together with the nucleotide sequences which are to be the subject of recombination is envisaged. Also envisaged are methods for performing the present invention wherein some of the homologous recombination system proteins are expressed in the host cell while the remainder are introduced into the cell in protein form. For example, expression within the host cell of *recE* and *recT* or functional derivatives thereof together with the coordinated administration of gam protein is envisaged. This aspect of the present invention should be understood to extend to the delivery of any one or more of the exonuclease, recombination protein or recBCD inhibitor or functional derivatives thereof.

"Derivatives" include fragments, parts, portions, chemical equivalents, mutants, homologues, analogues, mimetics from natural, synthetic or recombinant sources including fusion proteins. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences including fusions with other peptides, polypeptides or proteins.

The derivatives of said nucleotide sequences and expression products (referred to as "components") include fragments having particular epitopes or parts of the entire component fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, said components or derivative thereof may be fused to a molecule to facilitate its entry into a cell. Analogs of said components contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational

constraints on the proteinaceous molecules or their analogs. Derivatives of nucleic acid sequences may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules of the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-

bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carboethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid contemplated herein is shown in Table 1.

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine		Chexa L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva

D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp

D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

The method of the present invention is useful as a simple and efficient technique for allowing the introduction of any desirable modification at any position in a circular DNA clone such as a BAC or PAC, without unwanted re-arrangements. Modifications such as the introduction of selectable markers or specific mutations corresponding to those found to cause human pathology are examples of the use to which this method can be put. The method of the present invention reduces the need for transferring cloned circular DNA molecules such as BACs/PACs to other *E. coli* strains for homologous recombination. It is also reduces the need for the creation of special shuttling vectors. The procedure is simple enough that it allows repeated use on any single clone to form complex changes. The procedure can be modified to allow the inclusion of a reporter gene or any other desired sequence into circular nucleic acid molecules.

The creation of accurate cell and animal models for specific mutations necessitates minimal disturbance of normal gene structure. Specific mutation alterations to gene structure may be introduced using the recombination method of the present invention in a two-step procedure. First a PCR product carrying tetracycline or a cassette containing an antibiotic selectable marker linked to a second counter-selectable marker (*e.g.*, *ccdB*, *sacB*), is introduced into the target site, which is subsequently precisely knocked out in

the second step with a genomic fragment carrying only the desired modification. Correct products should be readily obtained by counter-selection for clones that have lost the tetracycline gene or the second selectable marker.[33, 39, 39, 43] Depending on the targeted sites for integration in the circular DNA molecule, these approaches allow for very sensitive assay systems for monitoring the impact of various sequence elements and mutations on the tissue and developmental specificity of gene transcription, splicing and expression from intact functional loci. These techniques facilitate the development of pharmacological approaches aimed at altering the expression of genes with increased tissue and locus specificity, as well as human artificial chromosomes, as a means of alleviating or curing a variety of human genetic diseases. The creation of accurate animal models with normal and mutant functional loci also facilitates gene therapy approaches involving gene supplementation or correction of the mutant locus.

Another aspect of the present invention contemplates a method for facilitating the homologous recombination of at least two nucleotide sequences in a host cell said method comprising the steps of:

(i) transforming a host cell, wherein the transformed host cell comprises a first nucleotide sequence encoding an exonuclease, a recombination protein and a recBCD inhibitor, or functional derivatives thereof, the expression of which exonuclease, recombination protein and gene encoding the recBCD inhibitor is modulatable, and said at least two nucleotide sequences; and

(ii) culturing said transformed host cell for a time and under conditions sufficient for expression of said first nucleotide sequence to occur wherein the expression products of said first nucleotide sequence facilitate the homologous recombination of said second nucleotide sequence with said third nucleotide sequence.

Preferably, said exonuclease is recE, said recombination protein is recT and said gene encoding a recBCD inhibitor is *gam*.

Even more preferably said at least two nucleotide sequences are a nucleotide sequence comprising an F-plasmid portion and a linear nucleotide sequence. Still more preferably, said nucleotide sequence comprising an F-plasmid portion is BAC.

Most preferably said host cell is DH10B or mutant or homolog thereof.

Still another aspect of the present invention contemplates a method of producing a microorganism useful for facilitating homologous recombination between at least two nucleotide sequences said method comprising genetically manipulating a host cell such that it is capable of expressing *recBCD,* modulatable levels of an exonuclease, a recombination protein and a gene encoding a recBCD inhibitor and said at least two other nucleotide sequences.

Preferably, said exonuclease is recE, said recombination protein is recT and said gene encoding a recBCD inhibitor is *gam.*

Even more preferably said at least two nucleotide sequences are a nucleotide sequence comprising an F-plasmid portion and a linear nucleotide sequence. Still more preferably, said circular nucleotide sequence comprising an F-plasmid portion is BAC.

Even more preferably said host cell is DH10B or mutant or homolog thereof.

Still another aspect of the present invention contemplates a cell capable of facilitating homologous recombination between at least two nucleotide sequences said cell comprising nucleotide sequences encoding an exonuclease, a recombination protein and a gene encoding a recBCD inhibitor, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, recBCD and said at least two nucleotide sequences.

Preferably, said exonuclease is recE, said recombination protein is recT and said gene encoding a recBCD inhibitor is *gam.*

Even more preferably said at least two nucleotide sequences are a nucleotide sequence comprising an F-plasmid portion and a linear nucleotide sequence. Still more preferably, said nucleotide sequence comprising an F-plasmid portion is BAC.

Even more preferably said cell is DH10B or mutant or homolog thereof.

Still another aspect the present invention contemplates nucleic acid molecules homologously recombined by the method of the present invention.

Preferably said modified nucleic acid molecules are modified BACs or modified PACs.

The present invention also extends to the use of said modified nucleic acid molecules in the treatment and/or diagnosis of patients. Methods of treatment include gene therapy regimens. The present invention also extends to methods of screening which utilise said modified nucleic acid molecules.

Accordingly, another aspect of the present invention contemplates a pharmaceutical composition comprising modified nucleic acid molecules generated by the method of the present invention together with one or more pharmaceutically acceptable carriers and/or diluents.

In a most preferred embodiment there is provided an expression vector comprising a genetic construct substantially as set forth in Figure 3 or functional derivative thereof.

Yet another aspect of the present invention is directed to a kit for facilitating, in a host cell, the homologous recombination of at least two nucleotide sequences said kit comprising compartments adapted to contain any one or more of nucleotide sequences encoding an exonuclease, a recombination protein, a recBCD inhibitor or functional derivatives thereof, and reagents useful for facilitating homologous recombination. Further compartments may also be included, for example to receive biological samples such as any one or more of the nucleotide sequences which are to be recombined, the

host cells or host cells already stably transformed with one or more of the nucleotide sequences which are to be recombined.

Still yet another aspect of the present invention is directed to a kit for facilitating, in a host cell, the homologous recombination of at least two nucleotide sequences said kit comprising compartments adapted to contain any one or more of an exonuclease, a recombination protein, a recBCD inhibitor, or functional derivative thereof and reagents useful for facilitating homologous recombination. Further compartments may also be included, for example to receive biological samples such as any one or more of the nucleotide sequences which are to recombined, the host cell or host cells already stably transformed with one or more of the nucleotide sequences which are to recombined.

The kits according to this aspect of the present invention should also be understood to extend to kits which comprise a combination of nucleotide sequences encoding one or more recombination system proteins or the recombination system proteins themselves.

Further features of the present invention are more fully described in the following non-limiting Figures and/or Examples. It is to be understood, however, that this detailed description is included solely for the purpose of exemplifying the present invention.

EXAMPLE 1

Inducible recE pathway of homologous recombination of PCR fragments into circular substrates in *E. coli* DH10B cells

The recE pathway of homologous recombination between linear and circular substrates is active in the *sbcA recBC* strains JC8679 and JC9604.[42-45] This pathway is *recA* independent,[46] since the *recT* gene appears to fulfil the role of *recA* in pairing of homologous sequences.[47] Precise homologous recombination of PCR fragments of a kanamycin resistance (Km^r) gene (PCRkan50) carrying 50bp flanking homology arms, into the backbone of the chloramphenicol resistant (Cm^r) pEBAC140 vector in these cells was obtained, to generate pEBAC140::kan50. However, it was not possible to transfer the 200kb pEBAC/148-globin clone from DH10B into JC9604 electrocompetent cells for subsequent modification, presumably due to host restriction differences between the two strains. Since BACs/PACs are normally maintained in DH10B cells, it is desirable to carry out any modifications directly in the DH10B cells carrying the BACs/PACs of interest, rather than having to transfer each BAC/PAC to another *E. coli* strain. However, electroporation of PCRkan50 into DH10B(pEBAC140) did not generate any Cm^rKm^r recombinants. It was also not possible to obtain recombinants with PCRkan140, a 1450bp PCR fragment carrying about 140bp flanking homology arms to the backbone of pEBAC140 in DH10B cells.

The pBAD24-recET plasmid which carries the *recE* and *recT* genes under the control of an inducible arabinose promoter, was electroporated into DH10B(pEBAC140) cells and clones carrying both plasmids were selected on ampicillin and chloramphenicol media. A single colony carrying both plasmids, DH10B(pBAD24-recET, pEBAC140), was used to prepare electrocompetent cells with L-arabinose induction during the growth of the cells. Again no Cm^rKm^r recombinant clones were obtained after electroporation of PCRkan50 into these cells.

A PCR fragment carrying the *gam* gene was inserted into the pBAD24-recET plasmid, to produce plasmid pGETrec (Fig. 3). With the placement of the *gam* gene under the same L-arabinose-inducible promoter as the *recE* and *recT* genes, the coordinate induction of the recE pathway and the inhibition of the recBCD nuclease activity on incoming linear dsDNA was sought. The inducible expression plasmid, pGETrec, was introduced into electrocompetent DH10B cells already carrying pEBAC140. Electrocompetent cells carrying both plasmids, DH10B(pGETrec, pEBAC140), were prepared with the standard protocol, except that L-arabinose was included in the growth medium from the initial inoculation of the cells. Electroporation of PCRkan140 into these cells resulted in almost 300 recombinants each from two independent electroporations (Table 1, Experiments 1, 2). Restriction enzyme digestion with *Xho*I or *Hin*dIII and PCR analyses of 12 randomly picked recombinants showed that every Cm^rKm^r recombinant had undergone homologous recombination of PCRkan140 into the correct target site on pEBAC140, without any evidence for unwanted rearrangements. This was confirmed by DNA sequencing of both regions of homology flanking the kanamycin gene. Although there was no selection for the pGETrec plasmid during growth of these recombinants on chloramphenicol/kanamycin media, variable amounts of this multicopy plasmid persisted with the single copy pEBAC140::kan140 plasmid in different clones.

EXAMPLE 2

Homologous recombination of PCRkan50 and PCRkan140 to the vector backbone of the 200kb β-globin clone pEBAC/148 in DH10B cells

Modification of the 200kb β-globin clone pEBAC/148 using PCRkan140, was attempted to determine if a large, single copy BAC clone could be modified using the same approach. pGETrec was electroporated into DH10B(pEBAC/148) cells. A clone carrying both plasmids, DH10B(pGETrec, pEBAC/148), was used to prepare electrocompetent cells. Cells were induced with L-arabinose for 40 minutes before they were harvested and made electrocompetent. Electroporation of PCRkan140 into these cells yielded more than 1000

Cm^rKm^r recombinants per electroporation (Table 1, Experiments 3, 4). No Cm^rKm^r recombinants were obtained after electroporation with PCRkan140 when the cells were induced with L-arabinose from the start of the culture prior to the preparation of electrocompetent cells. The prolonged overexpression of the *recE*, *recT*, and *gam* genes with L-arabinose induction is toxic to the DH10B cells. Cells carrying the 200kb BAC appeared to be more sensitive to prolonged induction than cells carrying the pEBAC140 vector.

20 independent Cm^rKm^r colonies were analysed by direct PCR on overnight cultures. Primers 140KA and 140KB were designed to generate a product of 1555bp from pEBAC/148::kan140. All 20 Cm^rKm^r colonies analysed generated the expected PCR product (Fig. 6). The parent pEBAC/148 clone was used as a control. The control PCR generated the expected fragment of 393bp from pEBAC/148 . None of the recombinant clones generated this fragment, which should have been detected if integration of PCRkan140 had taken place elsewhere in the bacterial chromosome. Thus, the PCR analysis indicates that the recombination event had taken place accurately in each of the 20 independent clones.

DNA was isolated from six of these Cm^rKm^r colonies. A large and variable amount of the multicopy pGETrec plasmid was recovered together with the single copy pEBAC/148::kan140 BAC and interfered with interpretation of restriction digests of the BAC. Re-electroporation of 1µl of DNA from four clones into DH10B cells resulted in many independent secondary Cm^rKm^r colonies from each clone. DNA was isolated from two independent secondary clones of each primary clone and analysed by pulsed field gel electrophoresis after digestion with *Not*I or *Xho*I (Fig. 7). All eight secondary clones contained only the large BAC, demonstrating that the kanamycin resistance marker was now an integral part of pEBAC/148 in these clones. *Not*I digestion (Fig. 7a) shows that all 8 secondary clones liberated the expected 185kb β-globin insert and did not suffer any gross rearrangements. There is also a slight but obvious increase in the size of the *Not*I-digested vector band from all eight clones compared to the *Not*I-digested vector band of the parent clone, pEBAC/148, corresponding to the expected change in size of the vector band

after integration of PCRkan140 into its target site. The same eight clones were further analysed after restriction digestion with *Xho*I (Fig. 7b). All showed the same restriction fragments as the control parent pEBAC/148 clone, except for a fragment of about 70 kb that is only observed in the parent pEBAC/148 clone. In the eight secondary clones, the 70kb fragment appeared to be cut into two fragments of about 30 and 40kb each. The 40kb fragment co-migrates with another fragment of about 40kb in length that is present in the parent pEBAC/148 clone, giving a significantly more intense band at this position for the Cm^rKm^r clones than the 40kb band generated by the parent pEBAC/148 clone. Since there is no *Xho*I site on the backbone of the parent vector, the 70kb fragment must be derived from the backbone of the vector and adjoining globin sequences. After integration of PCRkan140 into the vector backbone by homologous recombination, the 70kb fragment is cut into two smaller fragments at the single *Xho*I site which is present in the kanamycin gene.

These results confirm that integration of PCRkan140 through homologous recombination had taken place correctly into the target site on the backbone of the vector in all four primary Cm^rKm^r clones. Furthermore, it is clear that none of the clones displayed any instability through this procedure, indicating that transient induction of homologous recombination in DH10B cells with the pGETrec plasmid minimises the risk of unwanted rearrangements in BAC clones at least up to about 200kb in size. A more detailed analysis of the homology regions used for integration through homologous recombination was undertaken. Sequencing across the homology regions flanking the kanamycin gene in both pEBAC/148::kan140 clones and pEBAC140::kan50 clones did not reveal any deviations from the expected sequence.

PCR products incorporating homologous regions as short as 50bp have been shown to recombine with good efficiency in JC8679 or JC9604 *E. coli* strains.[42,43] This allows any fragment of interest to be amplified by synthesising 70-80-mers carrying 50bp homology to the target site (at the 5' ends of the primers) and used directly for targeting by electroporation. This approach was tested in the current system by electroporating PCRkan50 into DH10B(pGETrec, pEBAC/148) cells, which were only induced for 40 min

with L-arabinose during preparation. More than 100 Cm^rKm^r colonies were obtained from each electroporation (Table 1, Experiments 5, 6). Direct PCR screening of 20 independent overnight cultures from these Cm^rKm^r colonies, using primers 140KF and 140KR, generated the correct 1450bp product.

Although the frequency of recombinants was reduced about 10-fold using 50-mer targeting sites compared to 140-mer targeting sites, the accuracy of integration through homologous recombination is unaltered.

While no recombinants were obtained with the pGETrec plasmid in DH10B cells in the absence of L-arabinose induction, prolonged induction with L-arabinose is clearly toxic to the cells. Induction for more than 4hrs during the preparation of the electrocompetent cells and during recovery after electroporation appeared to reduce greatly the viability of the cells and did not yield any recombinants. On the other hand, electroporation of PCRkan50 into DH10B(pGETrec, pEBAC/148) cells that were induced for only 10 min with L-arabinose, generated about 10-fold less Cm^rKm^r than after 40 min induction. Although induction for 40 minutes yielded recombinant clones with high efficiency, modulation of the parameters can be used as a tool to modulate recombination efficiency.

EXAMPLE 3

Construction of pEBAC/148

The second generation pEBAC140 BAC/PAC cloning vector (Fig. 1) combines a number of features from the first generation PAC[24] and BAC[23] cloning systems, as well as the *oriP* and *EBNA-1* of Epstein Barr virus from the HAEC system.[48] It also contains a number of additional features, including a rare cutter multicloning region. pEBAC/148 (Fig. 2) was prepared from pEBAC140 by retrofitting a 185kb genomic fragment carrying the entire β-globin locus, using procedures based on the PAC cloning protocols.[49] Briefly, the RPCI 1 human total genomic PAC library[49] was screened by PCR primers from the 5'- and 3'-ends of the β-globin locus. A PAC clone carrying a 185kb genomic insert was identified (PAC148/β-globin) and shown to contain the entire β-globin locus (about 73kb), as well as

additional sequences at both the 5'- and 3'- ends. The 185kb genomic insert was isolated as a single fragment by *Not*I digestion, pulsed field gel electrophoresis (CHEF-DRII, BIO-RAD Labs., Hercules, CA) and gelase (Epicentre Technologies, Madison, WI) digestion. The pEBAC140 vector was also digested by *Not*I, dephosphorylated and gel purified before ligation to the purified 185kb globin fragment. Three independent pEBAC/148 clones were isolated after electroporation into DH10B cells. Analysis of the clones by *Not*I digestion and pulsed field gel electrophoresis showed that they all contained the intact 185kb β-globin genomic fragment, without any indication of rearrangement. Expression of the β-globin gene in pEBAC/148 was observed in a stable cell line in which pEBAC/148 is maintained in the episomal format[50].

EXAMPLE 4

Construction of the pGETrec inducible recombination vector

The *gam* gene of bacteriophage λ was PCR amplified using primers gam-F, 5'-AGGTAGGATCCACCATGGATATTAATACTGAAAC-3' (SEQ ID NO:1), and gam-R, 5'-ACTGAGGATCCTCGTTTTATACCTCTGAATCAATAT-3' (SEQ ID NO:2), from plasmid pTP223,[41] digested with *Bam*HI, and cloned into the *Bgl*II site of pBAD24-recET.[43] A clone with the correct orientation of the *gam* gene relative to the arabinose promoter was designated pGETrec (Fig. 3).

EXAMPLE 5

L-arabinose induction of the *recE, recT* and *λgam* genes and preparation of electrocompetent cells

Overnight cultures of DH10B cells (GIBCO-BRL, Gaithersburg, MD) carrying both pGETrec and either pEBAC140 or pEBAC/148 were diluted 50-fold into 250ml fresh LB medium containing 100μg/ml ampicillin and 12.5 μg/ml chloramphenicol. L-arabinose (SIGMA, St. Louis, MO) was added to a final concentration of 0.2% (w/v) when the OD600 of the cells reached 0.40 – 0.42. Electrocompetent cells were prepared by harvesting at OD_{600} 0.55 to 0.60 using the protocol recommended by the manufacturer. The

final cell pellet was resuspended in a total volume of 400 to 500µl of 10% glycerol. Cells were aliquoted to pre-chilled 1.5ml microfuge tubes (40µl/tube) and frozen in a dry ice-ethanol bath. The frozen cells were stored at -70°C.

EXAMPLE 6

Preparation of targeting DNA fragments PCRkan50 and PCRkan140

The kanamycin gene from vector pCYPAC2[49] was amplified with the following primers: kan50F 5'-ACCACATACGTTCCGCCATTCCTATGCGATGCACATGCTGTATGCCGGTACAAGAAATCACAGCCGAAGC-3' (SEQ ID NO:3) and kan50R 5'-TGAACTGATGGACTTATGTCCCATCAGGCTTTGCAGAACTTTCAGCGGTAGCGTGATCTGATCCTTCAACT-3' (SEQ ID NO:4). The first 50 nucleotides in each primer sequence (underlined) correspond to the sequence in the upper and lower strands of the pEBAC140 vector bordering the unique *Bst*1107I site, while the last 20 nucleotides of each primer correspond to sequences flanking the kanamycin gene in the pCYPAC2 vector. The PCR product, PCRkan50, is 1262bp long and carries the kanamycin gene and promoter region, as well as 50mer sequences at each end for targeting its integration through homologous recombination into the *Bst*1107I site on the pEBAC140 vector (Fig. 4). This was used to generate pEBAC140::kan50 by targeting the integration of PCRkan50 into pEBAC140 in *E. coli* JC8679 cells. PCRkan140 is 1450bp long and was generated by PCR from pEBAC140::kan50 by using primers 140KF 5'-ATCTGGGAAGTGACGGACAG-3' (SEQ ID NO:5) and 140KR 5'-CAGCATCGCAACCGCATCAG-3' (SEQ ID NO:6). PCRkan140 has 145bp and 143bp homologous targeting sequences at the 5'- and 3'-ends of the kanamycin gene respectively. PCR products were purified by gel electrophoresis, recovered through a QIAquick gel extraction kit (Qiagen, GmbH, Hilden, Germany) and quantitated.

EXAMPLE 7

Generation of recombinants

The overall procedure for the generation of recombinants is outlined (Fig. 5). Frozen stocks of 40µl of electrocompetent cells in microfuge tubes were thawed on ice and 200ng of PCRkan50 or PCRkan140 was added into each tube of cells. The mixture of DNA and competent cells were transferred quickly into a pre-chilled 0.2cm electrode gap cuvette (BIO-RAD Labs., Hercules, CA) and electroporated using a BIO-RAD Gene Pulser apparatus. Electroporation conditions were: 2.5kV, 200Ω, 25µF. One ml of LB medium was added to the cuvette immediately after electroporation, and the cells transferred into a 17x100mm FALCON polystyrene tube (BECKTON-DICKINSON Labware, Lincoln Park, NJ). The tubes were incubated in a 37°C shaker (220rpm) for 1.5 hours. Cells from each electroporation were spread onto LB plates containing 12.5 µg/ml chloramphenicol and 35µg/ml kanamycin to identify recombinants. Electroporation efficiency of each batch of competent cells was measured by an independent electroporation with 10ng of pZeoSV2 (Invitrogen, Carlsbad, CA) and plating serial dilutions of the electroporation mixture onto low-salt LB (pH7.5) plates containing 25µg/ml Zeocin. Total number of surviving cells after electroporation was measured by plating serial dilutions of the DH10B(pGETrec, pEBAC140) or DH10B(pGETrec, pEBAC/148) electroporated competent cells (without addition of DNA) onto LB plates containing 100µg/ml ampicillin and 12.5 µg/ml chloramphenicol. Percentage of recombination was measured as percentage of kanamycin- and chloramphenicol-resistant colonies per survivors after electroporation.

EXAMPLE 8

Analysis of recombinants

DNA from chloramphenicol- and kanamycin-resistant colonies was isolated from 2ml overnight cultures grown in LB medium containing 12.5µg/ml chloramphenicol and 35µg/ml kanamycin using a modified alkaline lysis miniprep method.[49] *Not*I- or *Xho*I-digested BAC DNA was analysed by pulsed field gel electrophoresis in a 1% (w/v) agarose gel (SeaKem, FMC Bioproducts, Rockland, ME), in 0.5x TBE buffer at 6V/cm and 14°C.

The Low Range and Midrange I PFG markers (NEB, Inc., Beverly, MA) were used as standards. PCR analysis of recombinant clones was performed directly on overnight cultures using the AmpliTaq DNA polymerase kit (Perkin Elmer Corp., Norwark, CT). One microlitre aliquots of recombinants (from 2ml overnight cultures grown in the presence of 12.5µg/ml chloramphenicol and 35µg/ml kanamycin) and the parent clone (grown in the presence of 12.5µg/ml chloramphenicol only), were added directly to the PCR reactions. The recombination product of pEBAC140 and PCRkan50 was designated pEBAC140::kan50. Similarly, the recombination products of pEBAC/148 with PCRkan50 or PCRkan140 were designated pEBAC/148::kan50 and pEBAC/148::kan140 respectively. PCR primers used to verify pEBAC140::kan140 and pEBAC/148::kan140 were 140KA, 5'-ATAAGCTCATGGAGCGGCGTAAC-3' (SEQ ID NO:7) and 140KB, 5'-GTTCCACATTTCCATATAAAGGCCA-3' (SEQ ID NO:8). PCR primers used to verify both pEBAC/148::kan50 and pEBAC140::kan50 were 140KF and 140KR (Fig.4). PCR products were resolved on a 1.5% (w/v) agarose gel. PCR products were sequenced at the recombination junctions using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences, Inc., Cleveland, OH).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

BIBLIOGRAPHY:

1. Jaenisch R. Transgenic animals. *Science* 1988; **240**: 1468-1474.

2. Brinster RL, *et al.* Introns increase transcriptional efficiency in transgenic mice. *Proc Natl Acad Sci U S A* 1988; **85**: 836-840.

3. Lamb BT, *et al.* Introduction and expression of the 400 kilobase amyloid precursor protein gene in transgenic mice [corrected] [published erratum appears in Nat Genet 1993 Nov;5(3):312]. *Nat Genet* 1993; **5**: 22-30.

4. Porcu S, *et al.* The human beta globin locus introduced by YAC transfer exhibits a specific and reproducible pattern of developmental regulation in transgenic mice. *Blood* 1997; **90**: 4602-4609.

5. Peterson KR, *et al.* Effect of deletion of 5'HS3 or 5'HS2 of the human beta-globin locus control region on the developmental regulation of globin gene expression in beta-globin locus yeast artificial chromosome transgenic mice. *Proc Natl Acad Sci U S A* 1996; **93**: 6605-6609.

6. Raguz S, *et al.* Muscle-specific locus control region activity associated with the human desmin gene. *Dev Biol* 1998; **201**: 26-42.

7. Choi T, Huang M, Gorman C and Jaenisch R. A generic intron increases gene expression in transgenic mice. *Mol Cell Biol* 1991; **11**: 3070-3074.

8. Vassaux G, Manson AL and Huxley C. Copy number-dependent expression of a YAC-cloned human CFTR gene in a human epithelial cell line. *Gene Ther* 1997; **4**: 618-623.

9. Gaensler KM, Kitamura M and Kan YW. Germ-line transmission and developmental regulation of a 150-kb yeast artificial chromosome containing the human beta-globin locus in transgenic mice. *Proc Natl Acad Sci U S A* 1993; **90**: 11381-11385.

10. Maas A, *et al*. The X-linked immunodeficiency defect in the mouse is corrected by expression of human Bruton's tyrosine kinase from a yeast artificial chromosome transgene. *Eur J Immunol* 1997; **27**: 2180-2187.

11. Peterson KR, *et al*. Transgenic mice containing a 248-kilobase human beta locus yeast artificial chromosome display proper developmental control of human globin genes. *Trans Assoc Am Physicians* 1993; **106**: 101-109.

12. Peterson KR, *et al*. Use of yeast artificial chromosomes (YACs) in studies of mammalian development: production of beta-globin locus YAC mice carrying human globin developmental mutants. *Proc Natl Acad Sci U S A* 1995; **92**: 5655-5659.

13. McCormick SP, *et al*. Mutagenesis of the human apolipoprotein B gene in a yeast artificial chromosome reveals the site of attachment for apolipoprotein(a). *Proc Natl Acad Sci U S A* 1995; **92**: 10147-10151.

14. Smith DJ and Rubin EM. Functional screening and complex traits: human 21q22.2 sequences affecting learning in mice. *Hum Mol Genet* 1997; **6**: 1729-1733.

15. Pavan WJ, Hieter P and Reeves RH. Modification and transfer into an embryonal carcinoma cell line of a 360-kilobase human-derived yeast artificial chromosome. *Mol Cell Biol* 1990; **10**: 4163-4169.

16. Frengen E, Wu C and de Jong PJ. Transfer of small YACs to E. coli as large circular plasmids. *Genet Anal* 1997; **14**: 55-59.

17. Bradshaw MS, Bollekens JA and Ruddle FH. A new vector for recombination-based cloning of large DNA fragments from yeast artificial chromosomes. *Nucleic Acids Res* 1995; **23**: 4850-4856.

18. Green ED and Olson MV. Chromosomal region of the cystic fibrosis gene in yeast artificial chromosomes: a model for human genome mapping. *Science* 1990; **250**: 94-98.

19. Green ED, Riethman HC, Dutchik JE and Olson MV. Detection and characterization of chimeric yeast artificial-chromosome clones. *Genomics* 1991; **11**: 658-669.

20. Larionov V, Kouprina N, Nikolaishvili N and Resnick MA. Recombination during transformation as a source of chimeric mammalian artificial chromosomes in yeast (YACs). *Nucleic Acids Res* 1994; **22**: 4154-4162.

21. Peterson KR, Clegg CH, Li Q and Stamatoyannopoulos G. Production of transgenic mice with yeast artificial chromosomes. *Trends Genet* 1997; **13**: 61-66.

22. Larionov V, *et al*. Specific cloning of human DNA as yeast artificial chromosomes by transformation-associated recombination. *Proc Natl Acad Sci U S A* 1996; **93**: 491-496.

23. Shizuya H, *et al*. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. *Proc Natl Acad Sci U S A* 1992; **89**: 8794-8797.

24. Ioannou PA, *et al*. A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. *Nat Genet* 1994; **6**: 84-89.

25. Hubert RS, *et al.* BAC and PAC contigs covering 3.5 Mb of the Down syndrome congenital heart disease region between D21S55 and MX1 on chromosome 21. *Genomics* 1997; **41**: 218-226.

26. Nechiporuk T, *et al.* A high-resolution PAC and BAC map of the SCA2 region. *Genomics* 1997; **44**: 321-329.

27. Wooster R, *et al.* Identification of the breast cancer susceptibility gene BRCA2 [see comments] [published erratum appears in Nature 1996 Feb 22;379(6567):749]. *Nature* 1995; **378**: 789-792.

28. Marshall E and Pennisi E. Hubris and the human genome . *Science* 1998; **280**: 994-995.

29. Venter JC, *et al.* Shotgun sequencing of the human genome. *Science* 1998; **280**: 1540-1542.

30. Venter JC, Smith HO and Hood L. A new strategy for genome sequencing. *Nature* 1996; **381**: 364-366.

31. Antoch MP, *et al.* Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. *Cell* 1997; **89**: 655-667.

32. Hejna JA, *et al.* Functional complementation by electroporation of human BACs into mammalian fibroblast cells. *Nucleic Acids Res* 1998; **26**: 1124-1125.

33. O' Connor M, Peifer M and Bender W. Construction of large DNA segments in Escherichia coli. *Science* 1989; 244: 1307-1312.

34. Sternberg N. The P1 cloning system: past and future. *Mamm Genome* 1994; **5**: 397-404.

35. Boren J, *et al*. A simple and efficient method for making site-directed mutants, deletions, and fusions of large DNA such as P1 and BAC clones. *Genome Res* 1996; **6**: 1123-1130.

36. Kim SY, *et al*. Modification of bacterial artificial chromosome clones using Cre recombinase: introduction of selectable markers for expression in eukaryotic cells. *Genome Res* 1998; **8**: 404-412.

37. Chatterjee PK and Coren JS. Isolating large nested deletions in bacterial and P1 artificial chromosomes by in vivo P1 packaging of products of Cre-catalysed recombination between the endogenous and a transposed loxP site. *Nucleic Acids Res* 1997; **25**: 2205-2212.

38. Yang XW, Model P and Heintz N. Homologous recombination based modification in Escherichia coli and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat Biotechnol* 1997; **15**: 859-865.

39. Messerle M, *et al*. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. *Proc Natl Acad Sci U S A* 1997; **94**: 14759-14763.

40. Jessen JR, *et al*. Modification of bacterial artificial chromosomes through chi-stimulated homologous recombination and its application in zebrafish transgenesis. *Proc Natl Acad Sci U S A* 1998; **95**: 5121-5126.

41. Murphy KC. Use of bacteriophage lambda recombination functions to promote gene replacement in Escherichia coli. *J Bacteriol* 1998; **180**: 2063-2071.

42. Oliner JD, Kinzler KW and Vogelstein B. In vivo cloning of PCR products in E. coli. *Nucleic Acids Res* 1993; **21**: 5192-5197.

43. Zhang Y, Buchholz F and Stewart A. Simplified DNA enginnering using recombination in E. coli. (1998). (submitted).

44. Kawaguchi S and Kuramitsu S. Homologous ligation: a novel cloning method. *Trends Genet* 1994; **10**: 420.

45. Kolodner R, Hall SD and Luisi-DeLuca C. Homologous pairing proteins encoded by the Escherichia coli recE and recT genes. *Mol Microbiol* 1994; **11**: 23-30.

46. Kowalczykowski SC, *et al*. Biochemistry of homologous recombination in Escherichia coli. *Microbiol Rev* 1994; **58**: 401-465.

47. Noirot P and Kolodner RD. DNA strand invasion promoted by Escherichia coli RecT protein. *J Biol Chem* 1998; **273**: 12274-12280.

48. Sun TQ, Fernstermacher DA and Vos JM. Human artificial episomal chromosomes for cloning large DNA fragments in human cells [published erratum appears in Nat Genet 1994 Dec;8(4):410]. *Nat Genet* 1994; **8**: 33-41.

49. Ioannou P and DeJong P: Construction of Bacterial Artificial Chromosome Libraries based on the modified P1 (PAC) system. In: Dracopoli NC, Korf BR, Moir DT, Morton CC, Seidman CE, Seidman JG, Smith DR (ed). Current Protocols in Human Genetics. John Wiley and Sons: New York, 1996, pp 5.15.11-15.15.24.

50. Westphal EM, et al. A system for shuttling 200-kb BAC/PAC clones into human cells: stable extrachromosomal persistence and long-term ectopic activation. *Human Gene Therapy* 1998; **9**: 1863-1873.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for facilitating homologous recombination between at least two nucleotide sequences said method comprising inducing recombination between said at least two nucleotide sequences in a host cell which is capable of expressing *recBCD* and nucleotide sequences encoding an exonuclease, a recombination protein and a recBCD inhibitor or derivatives thereof, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, by culturing the host cell under conditions sufficient to facilitate the homologous recombination of the at least two nucleotide sequences.

2. The method according to claim 1 wherein said two nucleotide sequences are a circular nucleotide sequence and a linear nucleotide sequence.

3. The method according to claim 2 wherein said circular nucleotide sequence comprises an F-plasmid portion.

4. The method according to claim 3 wherein said F-plasmid portion is a BAC.

5. The method according to claim 3 wherein said F-plasmid portion is a PAC.

6. The method according to any one of claims 1-5 wherein said exonuclease is recE or functional derivative thereof and said recombination protein is recT or functional derivative thereof.

7. The method according to claim 6 wherein said recBCD inhibitor is gam, P22Abc or SSB or functional derivative thereof.

8. The method according to claim 7 wherein said recBCD inhibitor is gam or functional derivative thereof.

9. The method according to claim 8 wherein the nucleic acid molecules encoding said recE, recT and gam are operably linked to an inducible promoter.

10. The method according to claim 9 wherein said nucleic acid molecules encoding said recE, recT and gam are operably linked to a common inducible promoter.

11. The method according to claim 9 or 10 wherein said promoter is L-arabinose.

12. The method according to claim 11 wherein said nucleic acid molecules operably linked to a common L-arabinose promoter correspond to the expression plasmid pGETrec.

13. The method according to any one of claims 1-12 wherein said host cell is *E. coli*.

14. The method according to claim 13 wherein said *E. coli* expresses recBCD.

15. The method according to claim 14 wherein said *E. coli* is the strain RR1, DM1 or DH10B.

16. The method according to claim 15 wherein said strain is DH10B.

17. A method for facilitating the homologous recombination of at least two nucleotide sequences in a host cell said method comprising the steps of:

(i) transforming a host cell, wherein the transformed host cell comprises a first nucleotide sequence encoding an exonuclease, a recombination protein and a recBCD inhibitor, or functional derivatives thereof, the expression of which exonuclease, recombination protein and gene encoding the recBCD inhibitor is modulatable, and said at least two nucleotide sequences; and

(ii) culturing said transformed host cell for a time and under conditions

sufficient for expression of said first nucleotide sequence to occur wherein the expression products of said first nucleotide sequence facilitate homologous recombination of said second nucleotide sequence with said third nucleotide sequence.

18. The method according to claim 17 wherein said two nucleotide sequences are a circular nucleotide sequence and a linear nucleotide sequence.

19. The method according to claim 18 wherein said circular nucleotide sequence comprises an F-plasmid portion.

20. The method according to claim 19 wherein said F-plasmid portion is a BAC.

21. The method according to claim 19 wherein said F-plasmid portion is a PAC.

22. The method according to any one of claims 17-21 wherein said exonuclease is recE or functional derivative thereof and said recombination protein is recT or functional derivative thereof.

23. The method according to claim 22 wherein said recBCD inhibitor is gam, P22Abc or SSB or functional derivative thereof.

24. The method according to claim 23 wherein said recBCD inhibitor is gam or functional derivative thereof.

25. The method according to claim 24 wherein the nucleic acid molecules encoding said recE, recT and gam are operably linked to an inducible promoter.

26. The method according to claim 25 wherein said nucleic acid molecules encoding said recE, recT and gam are operably linked to a common inducible promoter.

27. The method according to claim 25 or 26 wherein said promoter is L-arabinose.

28. The method according to claim 27 wherein said nucleic acid molecules operably linked to a common L-arabinose promoter correspond to the expression plasmid pGETrec.

29. The method according to any one of claims 17-28 wherein said host cell is *E. coli*.

30. The method according to claim 29 wherein said *E. coli* expresses recBCD.

31. The method according to claim 30 wherein said *E. coli* is the strain RR1, DM1 or DH10B.

32. The method according to claim 31 wherein said strain is DH10B.

33. A method of producing a micro-organism useful for facilitating homologous recombination between at least two nucleotide sequences said method comprising genetically manipulating a host cell such that it is capable of expressing recBCD, modulatable levels of an exonuclease, a recombination protein and a gene encoding a recBCD inhibitor and said at least two other nucleotide sequences.

34. The method according to claim 33 wherein said two nucleotide sequences are a circular nucleotide sequence and a linear nucleotide sequence.

35. The method according to claim 34 wherein said circular nucleotide sequence comprises an F-plasmid portion.

36. The method according to claim 35 wherein said F-plasmid portion is a BAC.

37. The method according to claim 35 wherein said F-plasmid portion is a PAC.

38. The method according to any one of claims 33-37 wherein said exonuclease is recE or functional derivative thereof and said recombination protein is recT or functional derivative thereof.

39. The method according to claim 38 wherein said recBCD inhibitor is gam, P22Abc or SSB or functional derivative thereof.

40. The method according to claim 39 wherein said recBCD inhibitor is gam or functional derivative thereof.

41. The method according to claim 40 wherein the nucleic acid molecules encoding said recE, recT and gam are operably linked to an inducible promoter.

42. The method according to claim 41 wherein said nucleic acid molecules encoding said recE, recT and gam are operably linked to a common inducible promoter.

43. The method according to claim 41 or 42 wherein said promoter is L-arabinose.

44. The method according to claim 43 wherein said nucleic acid molecules operably linked to a common L-arabinose promoter corresponds to the expression plasmid pGETrec.

45. The method according to any one of claims 33-44 wherein said host cell is *E. coli*.

46. The method according to claim 45 wherein said *E. coli* expresses recBCD.

47. The method according to claim 45 wherein said *E. coli* is the strain RR1, DM1 or DH10B.

48. The method according to claim 47 wherein said strain is DH10B.

49. A cell capable of facilitating homologous recombination between at least two nucleotide sequences said cell comprising nucleotide sequences encoding an exonuclease, a recombination protein and a gene encoding a recBCD inhibitor, the expression of which exonuclease, recombination protein and recBCD inhibitor is modulatable, and said at least two nucleotide sequences.

50. A cell according to claim 49 wherein said homologous recombination is performed in accordance with the method defined in any one of claims 1 to 32.

51. A nucleic acid molecule homologously recombined by the method described in any one of claims 1 to 32.

52. Use of the nucleic acid molecule of claim 51 in the manufacture of a medicament for the treatment of a disease in a mammal.

53. Use of the nucleic acid molecule of claim 51 in the diagnosis of a condition in a mammal.

54. A pharmaceutical composition comprising the nucleic acid molecule of claim 51 together with one or more pharmaceutically acceptable carriers and/or diluents.

55. A kit for facilitating, in a host cell, the homologous recombination of at least two nucleotide sequences said kit comprising compartments adapted to contain any one or more of nucleotide sequences encoding an exonuclease, a recombination protein, a recBCD inhibitor or functional derivative thereof, and reagents useful for facilitating homologous recombination.

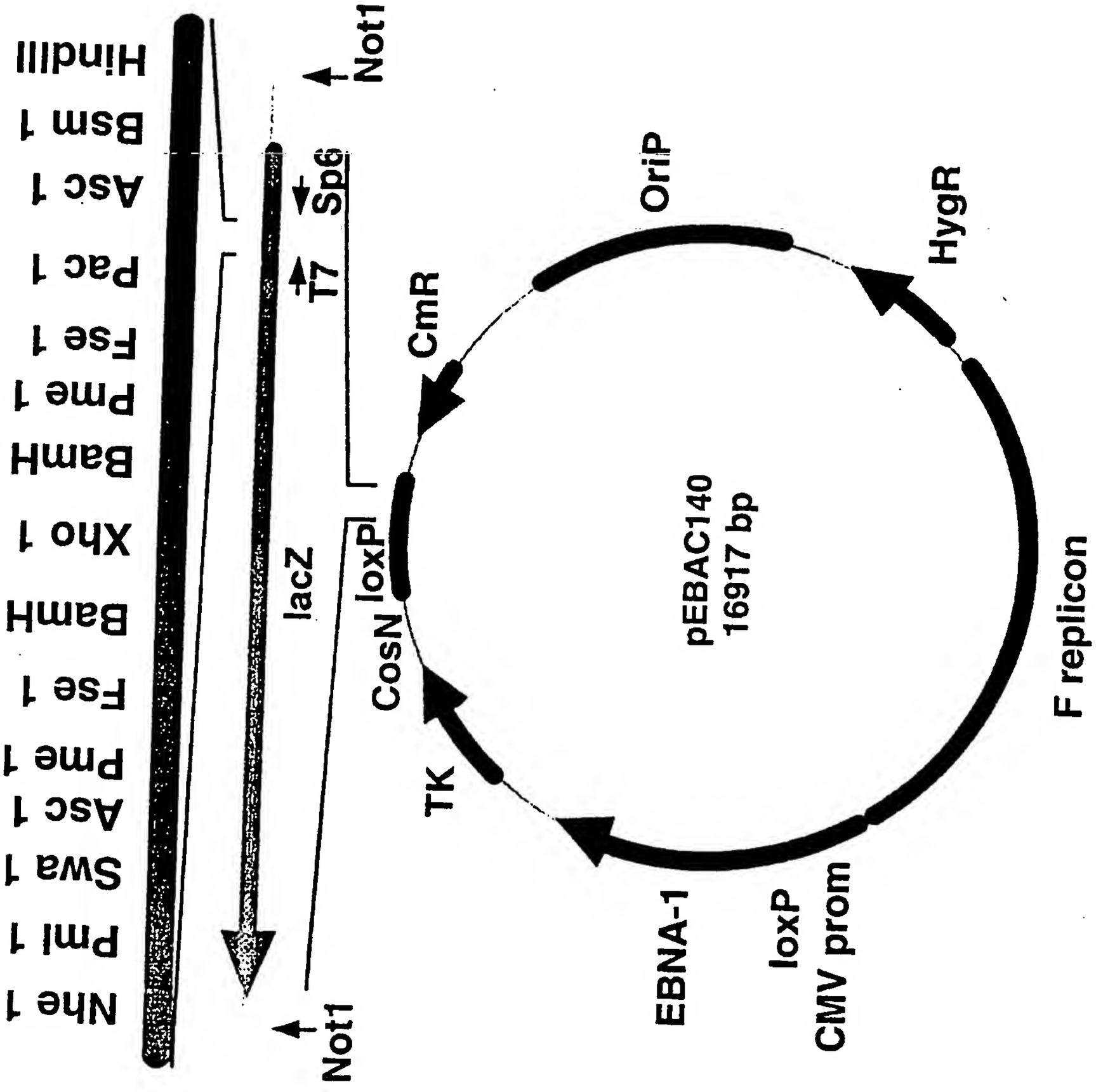
Not1
HindIII
BsiI 1
Bsm 1
Asc 1
Pac 1
Fse 1
Pme 1
BamH 1
Xho 1
BamH 1
Fse 1
Pme 1
Asc 1
Swa 1
Pml 1
Nhe 1
Not1
Sp6
T7
lacZ
CosN
loxP
CmR
TK
OriP
HygR
F replicon
pEBAC140
16917 bp
EBNA-1
loxP
CMV prom

Figure 1

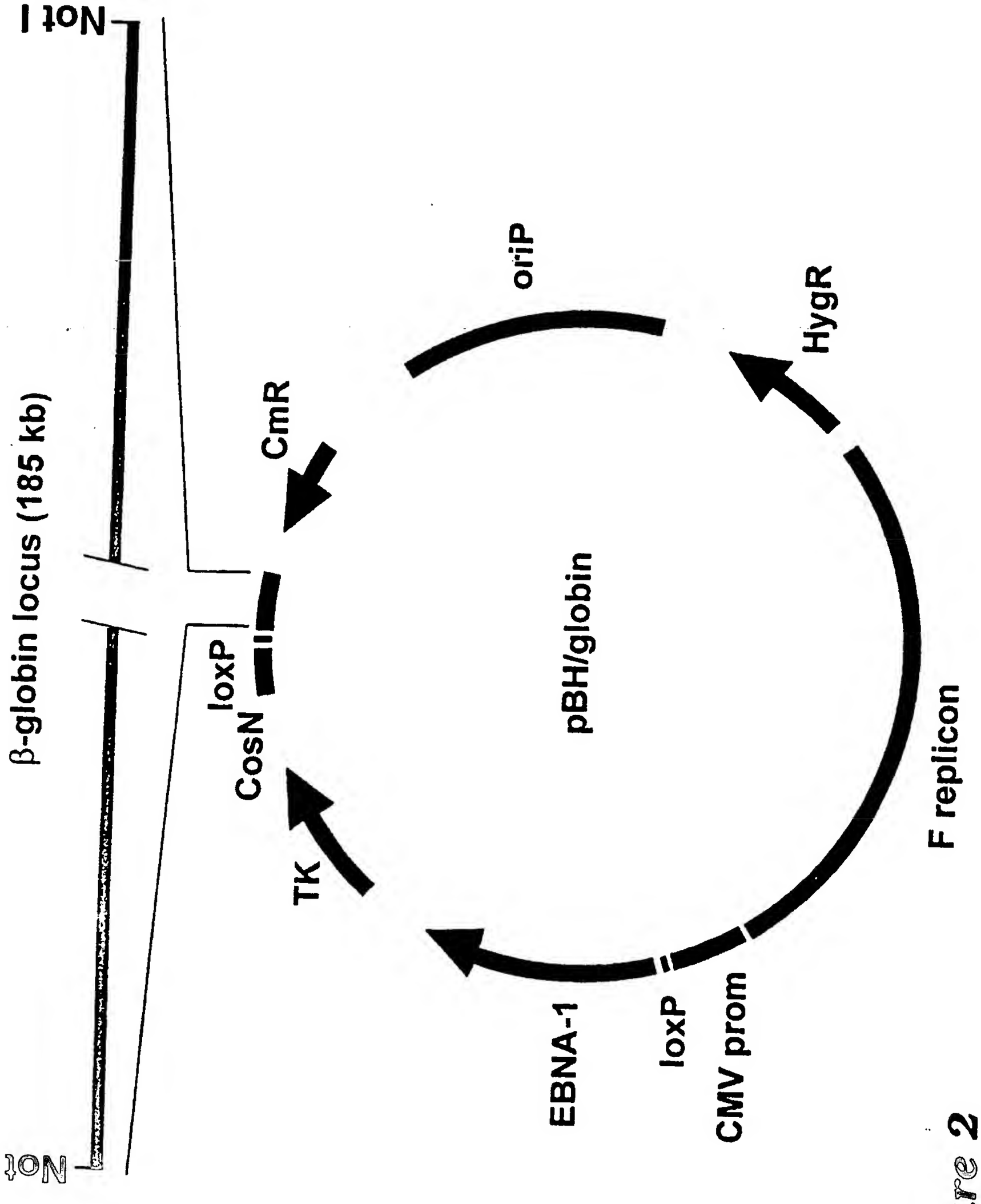
Not I
β-globin locus (185 kb)
CosN
loxP
CmR
TK
pBH/globin
oriP
HygR
F replicon
EBNA-1
loxP
CMV prom
Not I

Figure 2

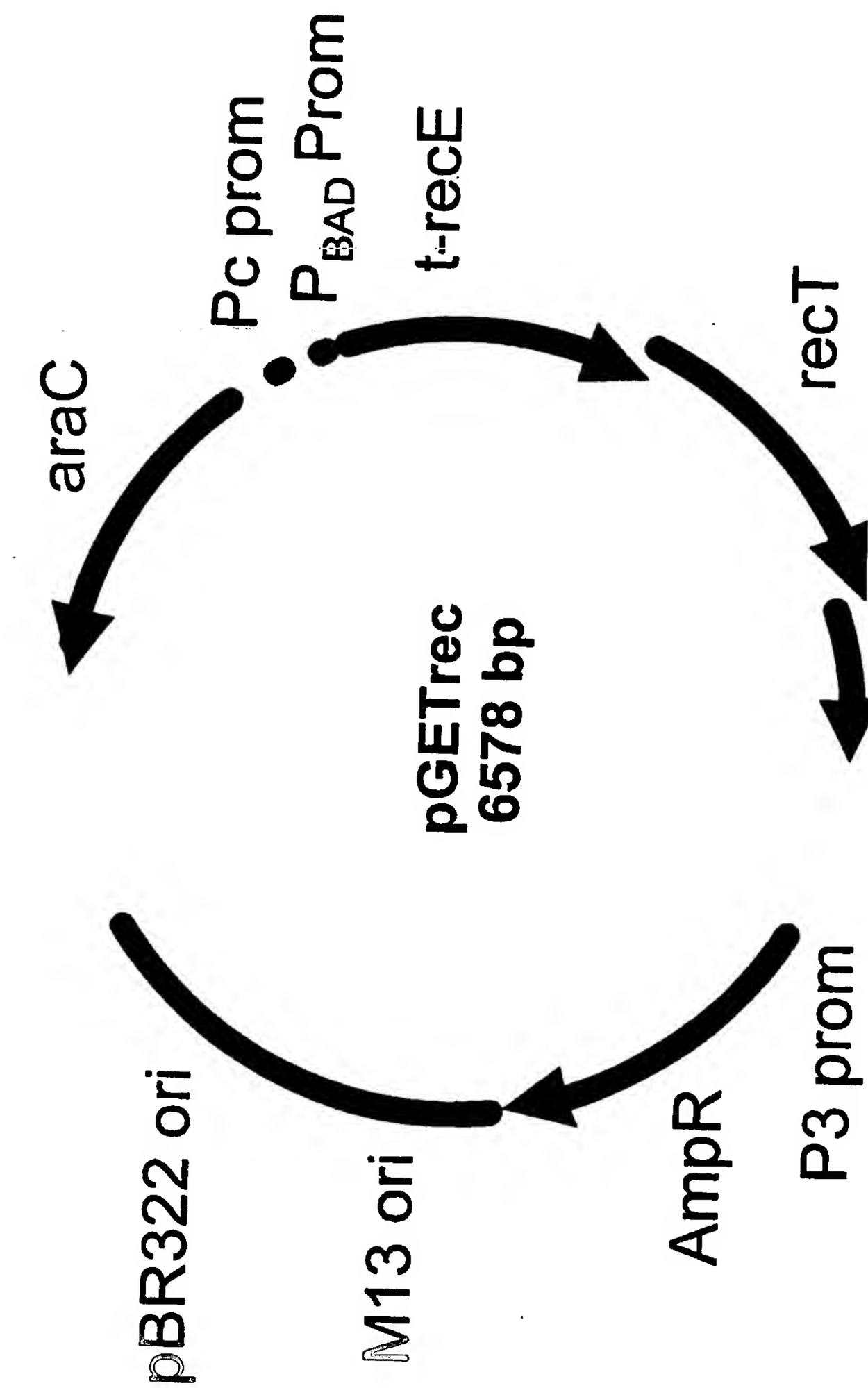
pGETrec
6578 bp
araC
Pc prom
P BAD Prom
t-recE
recT
gam
P3 prom
AmpR
M13 ori
pBR322 ori

Figure 3

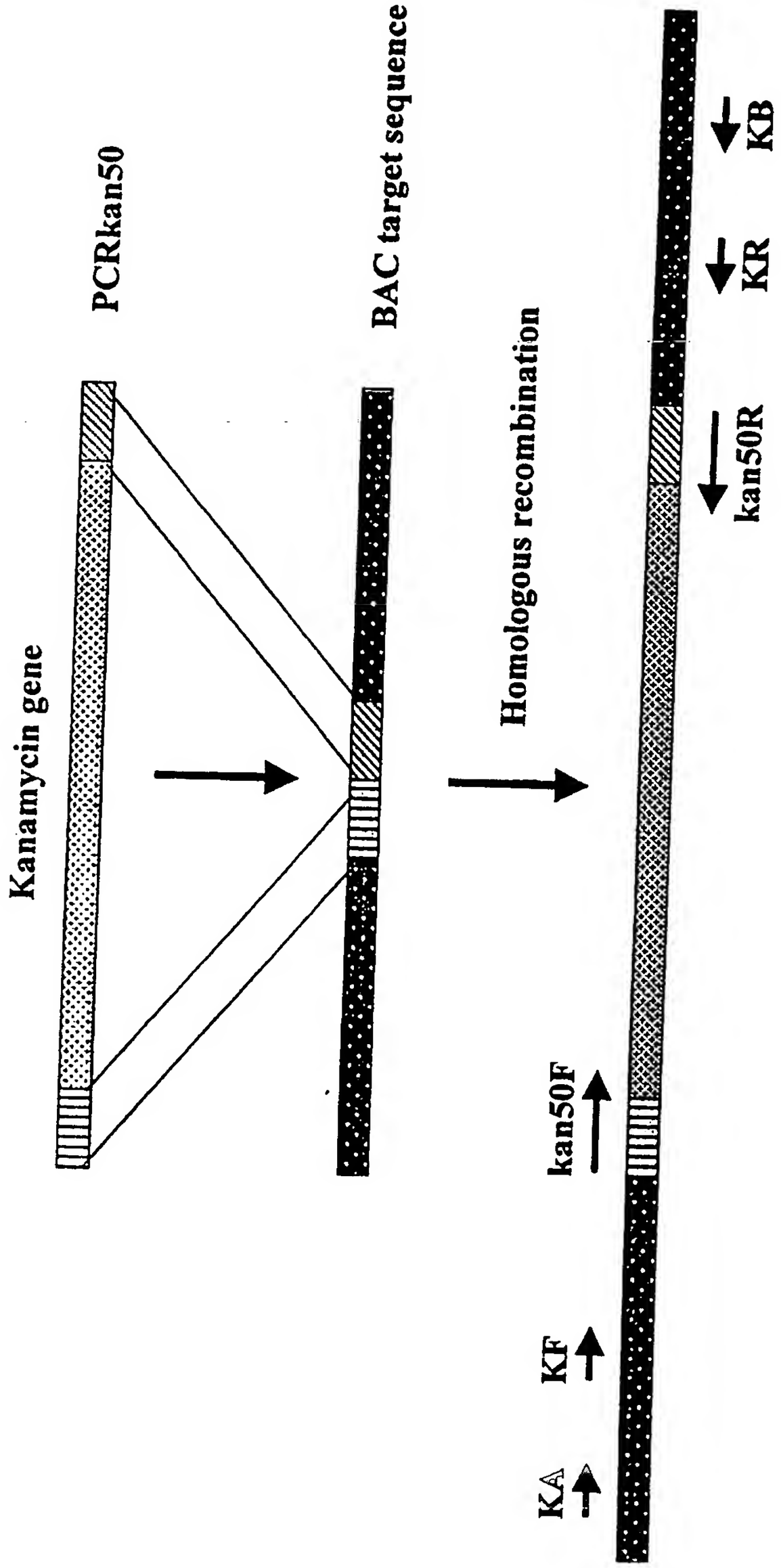
PCRkan50
BAC target sequence
Homologous recombination
KB
KR
Kanamycin gene
kan50R
kan50F
KF
KA

Figure 4

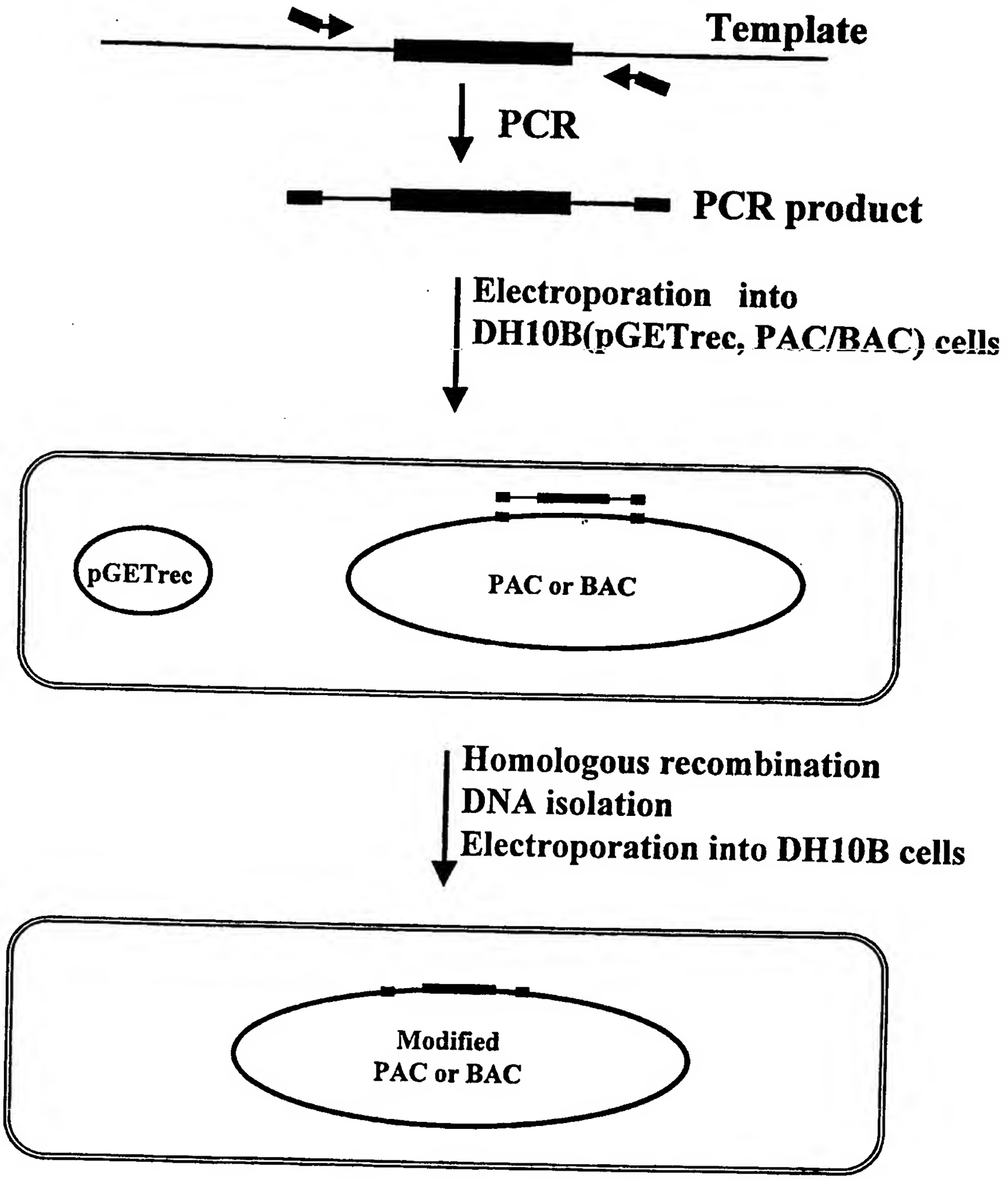
Template
PCR
PCR product
Electroporation into
DH10B(pGETrec, PAC/BAC) cells
pGETrec
PAC or BAC
Homologous recombination
DNA isolation
Electroporation into DH10B cells
Modified
PAC or BAC
Left Homology Arm
Sequence X
Right Homology Arm
Selectable marker

Figure 5

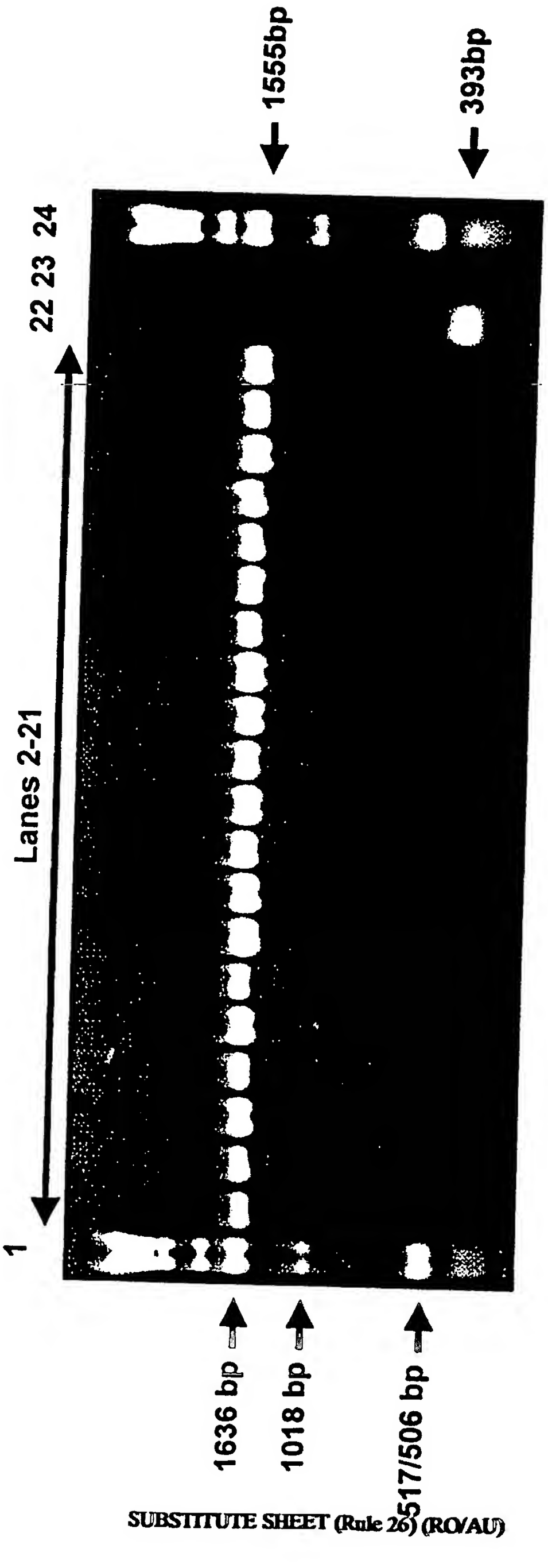

Figure 6

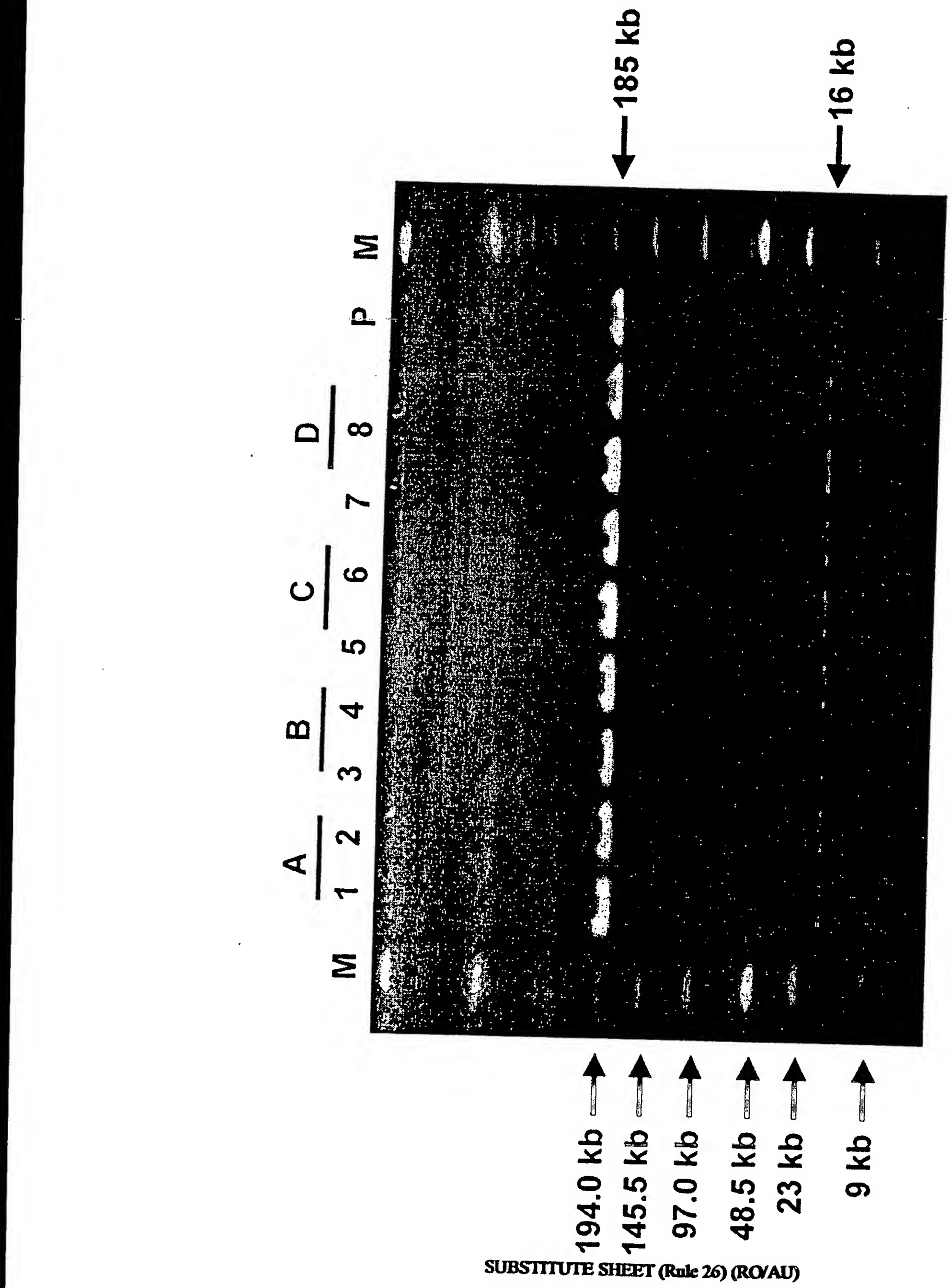
185 kb
16 kb
P M
A
1 2
B
3 4
C
5 6
D
7 8
M 1
194.0 kb
145.5 kb
97.0 kb
48.5 kb
23 kb
9 kb

Figure 7a

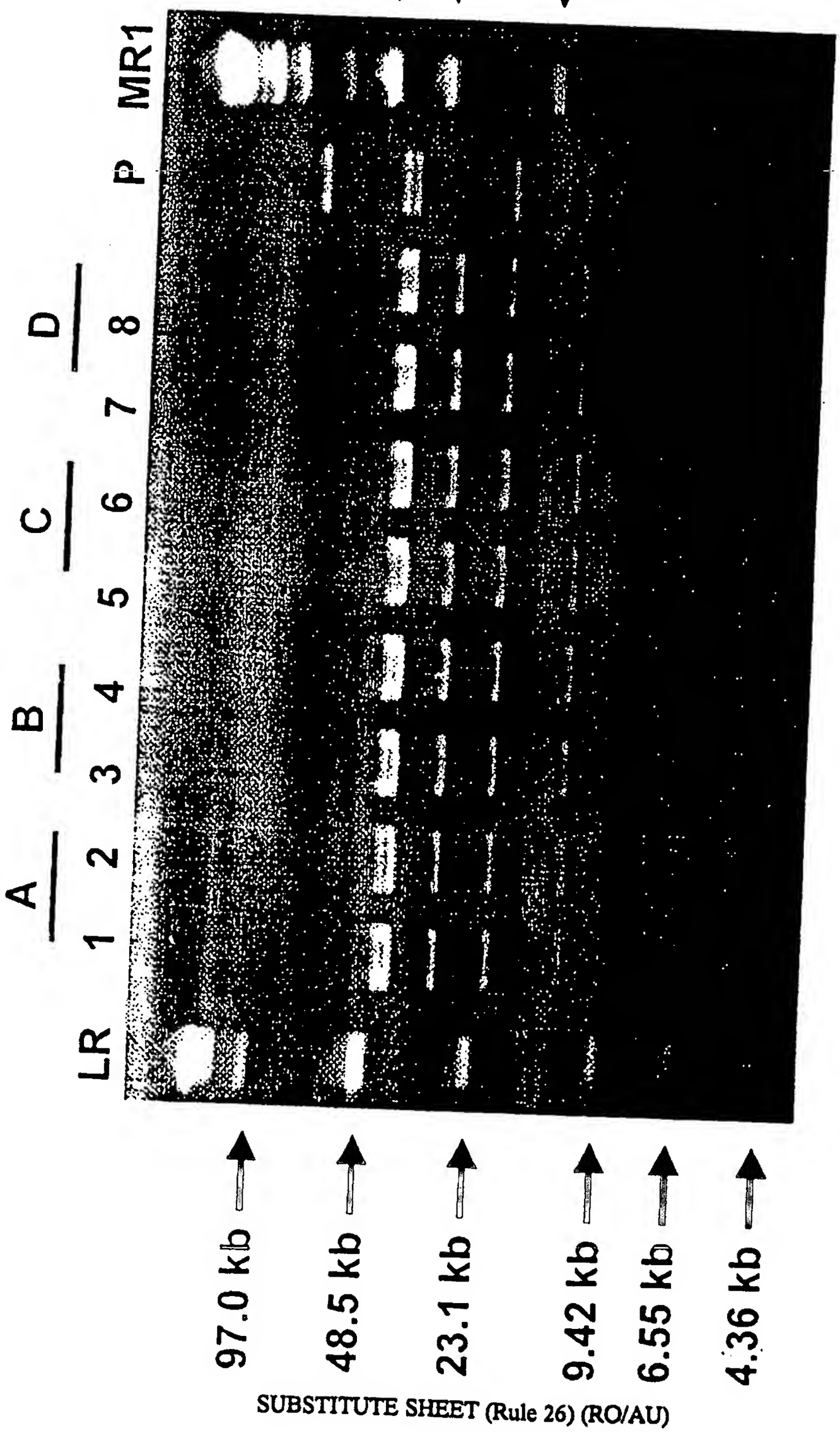
97.0 kb
82.0 kb
63.5 kb
48.5 kb
33.5 kb
15 kb
LR
A
1
2
B
3
4
C
5
6
D
7
8
P
MR1
97.0 kb
48.5 kb
23.1 kb
9.42 kb
6.55 kb
4.36 kb

Figure 7b

SEQUENCE LISTING

<110> THE MURDOCH INSTITUTE

<120> A METHOD OF RECOMBINATION AND AGENTS USEFUL FOR SAME

<130> 2219883/TDO

<140>
<141>

<150> PP6849/98
<151> 1998-10-30

<160> 8

<170> PatentIn Ver. 2.0

<210> 1
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PROBE/PRIMER

<400> 1
aggtaggatc caccatggat attaatactg aaac 34

<210> 2
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PROBE/PRIMER

<400> 2
actgaggatc ctcgttttat acctctgaat caatat 36

<210> 3
<211> 70
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PROBE/PRIMER

<400> 3
accacatacg ttccgccatt cctatgcgat gcacatgctg tatgccggta caagaaatca 60
cagccgaagc 70

<210> 4
<211> 71
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PROBE/PRIMER

<400> 4
tgaactgatg gacttatgtc ccatcaggct ttgcagaact ttcagcggta gcgtgatctg 60
atccttcaac t 71

<210> 5
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PROBE/PRIMER

<400> 5
atctgggaag tgacggacag 20

<210> 6
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PROBE/PRIMER

<400> 6
cagcatcgca accgcatcag 20

<210> 7
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PROBE/PRIMER

<400> 7
ataagctcat ggagcggcgt aac 23

<210> 8
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PROBE/PRIMER

<400> 8
gttccacatt tccatataaa ggcca 25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00835

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/87, C12N 15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, CHEMICAL ABSTRACTS (CA). Key words used (kw) See electronic database box below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
MEDLINE, USPM: Key words used (KW) See electronic database box below.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, USPM: KW Homologous recombination and (recBCD or BAC or PAC or artificial chromosome). Medline and CA: KW Homologous recombination and (recBCD or gam or Chi or P22Abc or SSB).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO, A, 99/29837 (EUROPÄISCHES LABORATORIUM FÜR MOLEKULRBIOLOGIE) published 17 June 1999. See whole document.	1-54
X	ZHANG Y *et al*: "A new logic for DNA engineering using recombination in *Escherichia coli*" NATURE GENETICS, vol 20 no.2 October 1998. See whole document.	1-54
X	BIANCO P R *et al*: "The recombination hotspot Chi is recognized by the translocating RecBCD enzyme as the single strand of DNA containing the sequence 5'-GCTTGGTGG-3'." PROC. NATL. ACAD SCI. USA vol 94 pp6706-6711 (1997) See whole document especially "Discussion".	55

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
18 November 1999

Date of mailing of the international search report
26 NOV 1999

Name and mailing address of the ISA/AU
AUSTRALIAN PATENT OFFICE
PO BOX 200
WODEN ACT 2606
AUSTRALIA
Facsimile No.: (02) 6285 3929

Authorized officer

J.H. CHAN
Telephone No.: (02) 6283 2340

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00835

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Murphy K C "λ Gam protein inhibits the Helicase and χ-stimulated recombination activities of *Escherichia coli* RecBCD enzyme" J. BACTERIOLOGY vol 173 no 18 p 5808-5821, Sept. 1991. See whole document.	55
X	KOLODNER R *et al* "Homologous pairing proteins encoded by the *Escherichia coli* recE and recT genes" MOLECULAR MICROBIOLOGY 11(1), 23-30 (1994). See whole document especially pages 24-26.	55
X	UMEZU K *et al* "Biochemical interaction of the *Escherichia coli* RecF, RecO, and RecR proteins with RecA protein and single-strand DNA binding protein" PROC. NATL. ACAD. SCI. USA vol 90 p. 3875-3879, May 1993. See whole document especially p 3876 and the "Discussion".	55

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/00835

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
WO	99/29837	AU	18771/99

END OF ANNEX